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**FIFRA SCIENTIFIC ADVISORY PANEL (SAP)**

OPEN MEETING

JUNE 18, 2003

DAY 2 OF 3

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Reported by: Frances M. Freeman

2

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**C O N T E N T S**

2

3 Proceedings.....Page 3

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1 DR. ROBERTS: I would like to open the second day of the  
2 session on Potential Developmental Effects of Atrazine on  
3 Amphibians. We are privileged to have today as our designated  
4 federal official Executive Secretary of the SAP, Larry Dorsey.

5 Larry, do you have any comments?

6 MR. DORSEY: No, I just welcome everyone again. We had a  
7 few requests for copies of materials from the public presenters. I just  
8 want to remind everyone that we are copying those materials now and  
9 they will be available in our public docket at Crystal Mall 2, within  
10 the next two or three days.

11 The phone number for the docket is on your agenda. All to the  
12 materials being used by the public or presented as part of the Public  
13 Presenters Presentations will be publicly available and will be in the  
14 docket.

15 Thank you.

16 DR. ROBERTS: Thank you, Larry.

17 In case we have any new folks in the audience today I would  
18 like to reintroduce the panel briefly and ask each member of the SAP  
19 Panel Session for this meeting to state their name, their affiliation  
20 and their area of expertise. We'll just go around the table just like we  
21 did yesterday, starting with Dr. LeBlanc.

4

1 DR. LeBLANC: Good morning. My name is Jerry LeBlanc and  
2 I am a professor in the Department of Environmental and Molecular  
3 Toxicology at North Carolina state University. My area of research  
4 specialization is endocrine toxicology.

5 DR. KLOAS: My name is Werner Kloas. I'm a Professor for  
6 Endocrinology at the University of Berlin. I'm also head of the  
7 Department of Inland Fisheries at the Institute for Freshwater  
8 Ecology and Inland Fisheries. My expertise is in endocrine disruption  
9 of amphibians concerning reproductive biology and thyroid systems.

10 DR. GREEN: I'm Sherril Green. I'm an Associate Professor in  
11 the Department of Comparative Medicine at Stanford University. My  
12 interest and expertise is in veterinary care and housing and husbandry  
13 of laboratory Xenopus and other species.

14 DR. COATS: I'm Joel Coats. I'm in the Department of  
15 Entomology at Iowa state University. I'm a professor of Entomology-  
16 Toxicology with specialization in pesticide environmental fate and  
17 the facts.

18 DR. DENVER: I'm Robert Denver. I'm an Associate Professor  
19 and Associate Chair of the Department of Molecular, Cellular and  
20 Developmental Biology at the University of Michigan, Ann Arbor.  
21 My expertise is in amphibian developmental neuroendocrinology.

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1 DR. GIBBS: My name is James Gibbs. I'm an Associate  
2 Professor in the Department of Environmental Enforced Biology, the  
3 State University of New York, Environmental Science and Forestry in  
4 Syracuse New York. My area of expertise is amphibian demography.

5 DR. RICHARDS: I'm Carl Richards. I'm a Professor of  
6 Biology at University of Minnesota, Deluth. I'm Director of the  
7 Minnesota Sea Grant College Program. My expertise is in the general  
8 area of aquatic ecology and landscape ecology.

9 DR. DELORME: My name is Peter Delorme. I'm a Senior  
10 Pesticide Researcher with the Canadian Government with the Pest  
11 Management and Regulatory Agency. My area of expertise is in  
12 ecotoxicology and risk assessment methods.

13 DR. SKELLY: My name is David Skelly. I'm an Associate  
14 Professor of  
15 Ecology at Yale University. My area of expertise is population and  
16 community of the ecology of amphibians.

17 DR. MATSUMARA: I'm Dr. Matsumara. I'm a Professor of the  
18 Environmental Toxicology. I also run the program called "The Center  
19 for Environmental Health Sciences. My areas of expertise are  
20 molecular toxicology and general toxicology relating to pesticide and  
21 pollutants.

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1 DR. THRALL: I'm Mary Anna Thrall. I'm a Professor in the  
2 Ecology of Veterinary Medicine and Biomedical Sciences at Colorado  
3 State University. My area of expertise is veterinary clinical  
4 pathology.

5 DR. ISOM: Gary Isom, Professor of Toxicology in the  
6 Department of Chemistry and molecular pharmacology at Perdue  
7 University. My area of expertise is neurotoxicology.

8 DR. HEERINGA: I'm Steve Heeringa, Research Scientist and  
9 Director of the Statistical Design Group at the Institute for Social  
10 Research at the University of Michigan. I'm a biostatistician and my  
11 specialization is in the design of population-based studies.

12 DR. ROBERTS: I'm Steve Roberts. I'm a Toxicologist,  
13 Professor in the Departments of Physiological Sciences and  
14 Pharmacology and Therapeutics and also a Director of the Center for  
15 Environmental and Human Toxicology, all at the University of  
16 Florida.

17 It is my pleasure to chair again today's session. I would like to  
18 welcome again, Dr. Steve Bradbury from Office of Pesticides  
19 Programs.

20 Good morning Dr. Bradbury.

21 DR. BRADBURY: Good morning. I just wanted to offer a few

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1 comments before we move on with more discussion from the public.  
2 Just a thank you to both the public commenters from yesterday and  
3 what we'll be hearing today as well as the very thoughtful and  
4 detailed deliberations and dialogue that the panel is having.

5 I'm very appreciative as is The Agency in the depth and rigor of  
6 these discussion. I think it is going to provide and is providing a very  
7 constructive and helpful input to the overall decision that we're  
8 working through. So, once again, just -- thank you very much for the  
9 in-depth and detailed discussions.

10 DR. ROBERTS: Thank you Dr. Bradbury. We're going to  
11 continue with our public comments. And before we begin, I would  
12 like to -- we have an extensive list of public commenters that would  
13 like to present. I would like to remind them that this panel is  
14 focusing on scientific issues related to our specific topic, which is  
15 development -- potential development effects of Atrazine on  
16 amphibians. It is not within the purview of this panel to debate issues  
17 of policy or law related to these topics.

18 Those are very important subjects certainly, but this is not the  
19 venue to raise those issues. I would like to request that each of our  
20 public commenters today restrict their comments to agencies -- I'm  
21 sorry -- to subjects or aspects of the problem that are specifically

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1 related to scientific issues. That's what we're really here to discuss.

2 Our next public commenters that were listed on our schedule,  
3 Dr. John Ashby and Charles Breckenridge, both on behalf of  
4 Syngenta. Are you here at the table and ready to go? Welcome, good  
5 morning.

6 DR. ASHBY: First of all I would like to thank the chairman,  
7 Larry Dorsey, the EPA and this SAP for the ability to speak to you  
8 today. I'm John Ashby. I'm a Senior Syngenta Fellow. I come from  
9 the Center of Toxicology, Laboratory of Syngenta in England.  
10 Charles Breckenridge, who will follow me is from the Greens  
11 Syngenta.

12 Now, what I want to do this morning is show you the history,  
13 the data and history as we, the primary registrants of this chemical,  
14 have seen it development.

15 The topic is rich in uncertainty. I hope that the comments I  
16 shall make will help the panel to focus their thoughts. I hope what I  
17 say will be a completely objective appraisal of the science.

18 The panel has copies of the slides I'm using in black and white,  
19 but a lot of them are in color and most -- some of them are animated.  
20 So, if possible, it would be better to be watching the screen.

21 A brief history. Atrazine was originally registered by Ciba.

9

1 Everything was relatively quiet until about '95, within the context of  
2 the present meeting -- was relatively quiet until report of potential  
3 reptile effects in the literature from Tim Gross, which I'll come back  
4 to later on.

5 In 1997, the Endocrine Panel, the Endocrine Panel was formed.  
6 This was mainly for two reasons. First of all, once it became clear  
7 that we may have to be working the area of reptiles and amphibians,  
8 that's beyond the scope of most toxicology laboratory's facilities.

9 There is -- actually, in these days there is another advantage to  
10 this process and that is if anybody has problems with data it's really  
11 having problems with their academic colleagues rather than industry,  
12 which is such an interesting advantage.

13 Now, there is some very strange company movements going in  
14 the late '90s, the Great Huebers of the late '90s. Ciba merged to form  
15 Novartis. Novartis and Zeneca Chemical Businesses merged and  
16 Syngenta was formed.

17 At that point, both Novartis and Syngenta brought with them  
18 quite extensive research facilities in endocrine disruption and those  
19 have been quite dramatically developed since by Syngenta. So, we  
20 have a core knowledge and scientific research facility in this area.

21 That brings us to today, in 2003. You heard the Endocrine

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1 Panel's discussion yesterday. The reason that we are giving another  
2 presentation from Syngenta today, it is slightly different.

3 First of all within our company, there is great scientific interest  
4 in what is going on. So, we're not just responding to data, we're  
5 actually very interested in the science that is going on.

6 As the primary registrants we are responsible, we have assumed  
7 responsibility for progressing this area amongst the various people  
8 who sell atrazine and we are assailed by data from all quarters. We  
9 are constantly synthesizing those data, because ultimately we have to  
10 decide on the safety or the potential hazards of this product and it is  
11 that process, that historical process, that I want to talk to you about.

12 Now amphibians are the subject of this meeting. I'm just going  
13 to give you a couple of slides about the mammalian toxicology  
14 because a huge amount of work has been devoted to it in recent years  
15 and it has been the subject of several SAPs of the EPA. We're  
16 in the situation where on the other effects we have established NOEL  
17 values.

18 Now, the basis of the -- essentially, all of them are made in  
19 toxicology of atrazine is on this hypothalamic pituitary gonadal axis.  
20 The main actor from the hypothalamus to the pituitary is gonadal  
21 releasing hormone, GnRH. That acts on a range of different

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1 cells on the pituitary which then release luteinizing hormone, poly-  
2 stimulating hormone, prolactin. These act on either the testes or the  
3 ovary and lead to the production of estradiol testosterone and dihydra  
4 testosterone.

5 Now, the biology -- the mammalian toxicology biology of  
6 atrazine is associated with its ability to affect the post generate and  
7 the hypothalamus for the release of GnRH. That leads to  
8 modifications of the levels of LH FSH and P prolactin and a range of  
9 effects.

10 The two that I have chosen to show you today, because they are  
11 relevant to this meeting are changes in serum hormone levels and in  
12 rodents, delayed puberty. One of the two ways this mechanism has  
13 been confirmed -- one is that the studies of Ralph Cooper show that if  
14 you add GrNH, you can overrule this block and reduce and  
15 essentially, obliterate the effects at the bottom.

16 And second is that recently my group have been looking at  
17 simulation of the effect by blocking the GrNH receptors in the  
18 pituitary with Antirelix (ph). This produces the same sort of changes,  
19 except they are much more marked.

20 If you get the dose where you really, truly do block the  
21 pituitary receptors, then you generate what you could describe as

1 Peter Pan Rats. They just fail to sexually mature. They stay as young  
2 rats, so while their body weight is going on, they are not sexually  
3 maturing -- such an extreme form of the mechanism of atrazine.

4 Now, this mechanism -- I'm sorry you can't quite read that.

5 There is very few small prints on these slides. This mechanism was  
6 raised yesterday as potentially applying to the props of the issues  
7 we're approaching with the frog. To date nobody has actually  
8 assessed that in any -- not assessed it at all.

9 In fact, the only place I can find mention of it is in the Tavera-  
10 Mendoza paper that we'll mention later on, where they looked in the  
11 pituitary for chromophilous (ph) and the conclusion was that the  
12 pituitary was not secreting hormones. But that, I think, and the panel  
13 will obviously know about this, it could well be associated with  
14 exposure carrying at stage 56 and the brain connect occurring at 58.

15 So, it may not then have been a valid experiment or valid  
16 assessment of the GrNH mechanism. To my knowledge, that's the  
17 only GrNH discussion that has been about atrazine in amphibians.

18 Now, one other piece of information, just to get it out of the  
19 way early on, is that when you see things like delayed puberty and  
20 hormonal changes, you immediately think of estrogenicity. There has  
21 been extensive literature, 32 publications as listed at the top there and

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1 many reviews.

2 I've just shown you here the data from one rather imminent  
3 group, Tim Zacharewski Group, where they went through a range of  
4 invitro assays, including antiestrogen and MCF-7 cells and the yeast  
5 assay. They did the uterotropic assay and some of the markers of it  
6 such as PR levels and peroxidase levels. And they also did rather  
7 definitive anti-yeast assays.

8 Now, there is no sign of activity. I think one of the few certain  
9 things in this area is that atrazine is not an estrogen or antiestrogen. I  
10 saw in one of the white papers somebody had noticed that in the  
11 uterotropic assay, although there is no increase in uterus weight, there  
12 is a small decrease, a significant decrease in uterus weight.

13 That is actually, if we just go back, that's exactly what you  
14 would expect. Antirelics (ph), by blocking the pituitary, actually  
15 reduces uterine weight in rats. It is because even prepubertal animals  
16 have some estradiol, probably being made by the adrenal gland.

17 If you block any production of estradiol, you actually reduce  
18 your uterine weight. I think that is a reflection of the GrNH. That is  
19 in a way confirmed by the formal antiestrogen assay at the bottom of  
20 that slide there, where there is no sign of antiestrogen activity.

21 Tim Zacharewski's quote is the "Reported Effects of atrazine

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1 and not mediated by ER."

2 Now, before our recent interest in amphibians there were no  
3 alerts to problems in the area. And I'll just show you three things.  
4 First of all there were a range of 4 fish full life cycle studies  
5 conducted in three species, prolonged exposure.

6 These studies, of course, involve the generation of an F-1 and  
7 evaluation of its reproductive capacity and there is no signs of  
8 anything problematic there in those fish studies. Those data could be  
9 made available to the panel as white paper should you wish them.

10 Secondly, from the available studies, Avenin studies, there is  
11 no evidence of untoward effects. There is a range of effect studies  
12 where you look at embryotoxicity growth. Again, those studies  
13 showed no activity.

14 So, that brings us to one of the first simple conclusions,  
15 atrazine does not seem to induce gross developmental effects in fish,  
16 birds or early stage xenopus. So, I suppose in public understanding of  
17 what we're discussing here, this discussion is nothing to do with the  
18 deformities of frogs in the so-called Minnesota frog syndrome. I  
19 think there is a very clear divide in that discussion and what we're  
20 discussing today.

21 Now, I mentioned early on that one of the first observations

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1 that made us set up the panel actually, eventually, was Tim Gross in  
2 '95, showing that atrazine effected turtle eggs following painting of  
3 the eggs with atrazine dissolved in ethanol.

4 The actual data that we're aware of in '95 is shown here. This is  
5 percentage of males. This is done under temperature conditions  
6 which should be producing males. Those three doses of atrazine  
7 produced a depression in the male ration -- male sex ratio, which was  
8 statistically significant.

9 Now, that panel which formed partially in response to those  
10 data, some of the early initiatives -- first of all the panel conducted a  
11 review of atrazine and considered there were insignificant risk to  
12 aquatic species but they recognized at an early stage the need for  
13 additional data on amphibians and reptiles.

14 The panel -- one of the first commissions was a study on  
15 amphibian metamorphosis gonadal and laryngeal development, which  
16 was start -- which was conducted by Dr. Hayes and Noriega. Over the  
17 initial range of 0.1 to 25 parts per billion of atrazine. Really, the  
18 results of that commission and some subsequent and some  
19 independent studies that formed the basis of today's review.

20 I think it is important to say that wildlife studies over the  
21 period we're looking at have been conducted against a very fluid

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1 background, a very rapidly developing science.

2 We're all aware of that stack and the various validation  
3 committees which are actually ongoing now and the vast amount that  
4 has been done. I'm not too sure that they are very near to a validated  
5 frog protocol yet. So, we're in changing times. Everybody is working  
6 very hard.

7 Another problem is the significance of most of the biomarkers  
8 we are considering is quite unclear with the reproductive significance  
9 such as shifts in hormone levels and the relationship of biomarkers to  
10 individual function is again uncertain. So, we're still not sure what  
11 testicular oocytes mean, for example.

12 Certainly, extrapolation from the individual to the problem is --  
13 to the population is very uncertain.

14 Now, these problems are still with us. They will be with us  
15 probably for the next two or three years, at least.

16 Now, the review of the talk. What I'm going to concentrate in  
17 the rest is I try and synthesize at the data you are considering, is the  
18 changes in sex ratio changes in laryngeal size, laboratory studies on  
19 frog gonads -- frog and toad gonads, field studies -- considering a  
20 brief consideration of a the aromatase induction hypothesis and  
21 conclusions and recommendations.

1 I would only talk to the aromatase. Charles Breckenridge will  
2 take it in more detail to try and indicate to you the great developments  
3 that are going on in this area at the moment.

4 Now, let's look first of all at the sex ratio that started this  
5 whole thing off. This is the one you have just seen. That was a  
6 painting in ethanol. Tim Gross repeated that experiment in turtles  
7 and this time used drenching and because there is obviously, some  
8 uncertainty about the differential amounts getting in ethanol or water,  
9 there is a high dose in the repeat study.

10 This time there is non significant change; there is no change at  
11 all.

12 Tim Gross also did some alligator eggs and there was no  
13 significant effect on sex ratio and as part -- later on I will talk about  
14 Lou Gillette alligator eggs, but as part of that study, he also  
15 determined no change in sex ratio in the alligators that were treated  
16 again in ethanol. The dose-range there is done as a straight line,  
17 because it is actually three logs further to the right. They are very  
18 high-dose levels. I will show you what that means later on.

19 Then there is a temperature dependence species for sex ratio.  
20 There is a range of xenopus -- not the xenopus, but the Hecker study  
21 from John Giesy's lab. There is Carr study, there is a Hex study and

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1 there is a Hecker study in rana.

2 So, there is a row of nonsignificant changes, in fact no changes.  
3 This actually is illustrated, but one of the problems in this area, you  
4 can get a positive response and it takes an inordinate amount of  
5 energy -- and energy and effort to actually decide what the truth is.  
6 In this case it doesn't appear to be any ability of atrazine to change  
7 sex ratios in reptiles or amphibians.

8 As we're talking about mode of action, I'm trying to dissect  
9 where we need a mode of action. It is still not too clear to me where a  
10 mode of action will be applying. It certainly isn't in this area.

11 Now, I mentioned earlier the initiative of the panel  
12 commissioning, Hayes and Noriega, to start looking at the frogs.  
13 There was a draft final report which was actually never issued, but  
14 which has been made available to the EPA, which was delivered to  
15 Syngenta in 2000.

16 There are several conclusions that we're going to follow up.  
17 One of them was reduce larynx muscle size at and greater than one  
18 part per billion of atrazine.

19 Now, the data that we had at that time from Hayes -- Dr. Hayes,  
20 I should call him Hayes from now on, I can't keep saying Dr. Tyrone  
21 Hayes, Professor Tyrone Hayes. So, if you'll excuse me, I know you

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1 are here Dr. Hayes, if you will accept me calling you Hayes from now  
2 on.

3 For simplicity I have just shown you the male muscles here.  
4 The female muscles are marginally smaller and run along, in fact,  
5 they are just below this red line. In all of these experiments, the  
6 female muscle is lighter.

7 Dr. Hayes had shown earlier, in his earlier studies that  
8 dihydrotestosterone produced an increase in this muscle size and  
9 that's consistent with it being an androgenic model. I don't believe  
10 there is a concurrent DHT in this study.

11 The first repeat was from Dr. Carr's lab and there was no  
12 significant change. The female muscle, of course, was lighter. There  
13 was a DHT positive control in this experiment and the cross section  
14 area was up about .31 to .33, exactly where the box is.

15 There is also a second study by Hecker. Again in xenopus and  
16 again, no significant effects on the male muscle. And Hecker also had  
17 a DHT positive control which is sitting exactly in the same place  
18 which is why it is superimposed on that box.

19 Now, the original reduction in muscle size is very small. There  
20 is no sign of it in the two repeats. But the first thing that strikes you  
21 about these data are the muscles per se are larger, it's rather an

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1 expanded scale. So, it is not as bad as it looks, but in the two repeat  
2 studies the muscles are larger.

3 This panel is aware of the potential reasons for that, because  
4 you were discussing it yesterday. There may be some association.  
5 First of all there is no obvious relationship to body weight. There is  
6 quite a big difference in body weight between the Hecker and the Carr  
7 studies and it doesn't correlate with those different muscle weights.  
8 We're not aware of the weights of the Hayes animals. Body weight  
9 might be an effect.

10 There is also the stage delay that was mentioned yesterday in  
11 these two repeat studies, because of co tree conditions and Kelley  
12 was mentioning the grow-out phenomena and attenuation of this effect  
13 as the animals mature. There may be something to find out in that.

14 The take-home message is the effect could not be repeated. The  
15 conditions of the experiment may illuminate why the muscle was  
16 larger and perhaps even why it can't be repeated in these experiments.

17 If the effect is limited to this stage specificity, and is  
18 attenuated by grow-out, then the effect itself becomes somewhat  
19 questionable in as much as tadpoles are never asked to croak. It is an  
20 interesting area and it is one for the panel to consider.

21 The conclusion at the moment is that the two studies have failed

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1 to reproduce these reported effects on laryngeal size. And at the  
2 moment until they are confirmed and if it's decided worthy of  
3 confirming them there is no need for mode of action.

4 Obviously, if they are repeat studies it sounds like it would be  
5 much better to be counting filaments and distinguishing between  
6 hydrotropy and hyperplasia as was mentioned yesterday.

7 Now, another conclusion the draft final report from Hayes and  
8 Noriega was there is no abnormal, undifferentiated or intersexual  
9 gonads observed in any of the treatment controls. However,  
10 subsequent reports from Hayes Lab and other studies have indicated  
11 some positive effects and that's what we're now going to look at and it  
12 is one of the main topics of this meeting.

13 A note in this, because it will have hit you already, as you look  
14 through the data, in all of the available amphibian laboratory and  
15 field studies of gonadal abnormalities, there are major qualitative,  
16 quantitative and dose response inconsistencies. They just hit you the  
17 moment you start looking at this data.

18 Underneath that on numerous differences and experimental  
19 design and methods species study and more logical terminology  
20 employed. Now, a lot of these -- whenever anybody tries to repeat an  
21 experiment they assume all the variables going to build in, because

22

1 they've always done it and not going to influence the outcome.

2       There is only when you have an outcome difference that you  
3 start having to look back and wonder which of these many small  
4 changes are the actual cause of the disagreement.

5       One of the biggest problems are these definitions. Now, it is  
6 rather a complex slide so, I won't put it all up. I will just mention a  
7 new things that everybody is using their own terminology and  
8 individuals change their terminology with time and we certainly over  
9 the last few months, because of the importance of this, have made a  
10 determined effort to try and understand this terminology and I'm  
11 afraid we can't.

12       There are many uncertainties about what people mean by what  
13 they say. At the moment it is a veritable tower of fable. We're all  
14 talking to each other in different languages. The most important thing  
15 to sort out is terminology in this whole area.

16       So, for example, hermaphroditism, how is it related to intersex,  
17 how is it related to mixed sex, how is this continuous related to  
18 gonadal abnormalities related to segmented testes.

19       I won't go into it except you have these data, these definitions  
20 and they may be of value to you.

21       Now, the lab studies, I'm going to build these up for you.

1 Again, it will be better to actually watch on the screen, because one of  
2 the problems with multiple buildup slides is that the last slide is the  
3 one that Xeroxed and that often bears no relationship to the earliest  
4 slides. That's the big disadvantage to power point.

5 This first slide is from the draft report on the data or in that  
6 draft report which the panel has. The terminology used there is  
7 interspersed by Hayes and Noriega's gonadal abnormalities.

8 Now, the first -- and this is the PNAS paper -- the first paper,  
9 the first data gained from Hayes and Noriega are these ones here. The  
10 dose is now extended up to 200 parts per billion and the significance  
11 across the whole-dose range. This is now referred to as a  
12 hermaphrodite or multi-testes. So, the terminology is different in that  
13 first draft report.

14 These data strikers are immediately interesting. They are large  
15 and interesting in red. That's why I put them in red. The most  
16 interesting thing is the absence of a dose response. Over all of these  
17 doses, the data reported in PNAS as between 16 and 20 percent. The  
18 requirement for dose response is one of the primary needs in science  
19 when you are deciding what is going on.

20 When you have a plateau and if it really is a plateau and is a  
21 confirmable plateau, then you are not talking about a dose related

1 effect, you are talking about the acquisition of a permissive condition  
2 where the chemical has done something which enables something to  
3 happen and the chemical can't do anymore.

4 You have turned a switch. Whether or not that is what is going  
5 on here I'm not sure. There is a great need to repeat this and to study  
6 dose response and to really confirm if it is a plateau. And the greater  
7 the effect you see the greater need is to repeat.

8 There is also a need in a situation with data set is important as  
9 this influential as this to actually have access to the base data so that  
10 people can do their own statistical manipulations and in the PNAS  
11 paper we just got the statement, 16 to 20 percent across the whole  
12 dose range. So, it's actually not very helpful in the scientific sense.

13 So, although it is dramatic, it is very difficult to know what to  
14 do with it. In the scientific sense, you just stand back and say, wow,  
15 that's interesting.

16 Now, the first attempt to repeat this was in Carr's laboratory.  
17 There was a significant effect of 25. This is now -- this was discussed  
18 yesterday. This is gonadal abnormalities. Because of the effect of  
19 25, partially because of the effect of 25 and the effect wasn't very  
20 strong, the experiment was repeated in John Giesy's lab, the Hecker  
21 study, and no effects were seen there or no significant effects seen

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1 there. You are aware of a potential problem with that study.

2 Quite amazingly, in the -- in the ethanol controls there was no  
3 atrazine, but there is this very low-dose level of 0.1 atrazine parts per  
4 billion atrazine for some reason is in the water controls. The reason  
5 for that has still not been resolved. It is a log below the lowest dose  
6 of atrazine evaluated in this study.

7 Although that in a way does certainly weaken the study,  
8 because people don't like -- nobody as a scientist likes a contaminated  
9 control. It is in my view when I first heard about it I was quite  
10 dismayed. To my mind that meant that's the end of that study.

11 When you look at it that should -- the presence of a log lower  
12 in one of the controls should not have the ability to remove effects of  
13 high doses or orders of magnitude higher. It is imperfection but I  
14 don't think it shouldn't lead to the dismissal of the data. That's my  
15 view.

16 Also using this discontinuous terminology of these blue  
17 columns, DuPreez did microcosm study and found nothing.

18 Now, the terminology problem, again, just recurs throughout  
19 this talk. And the next set of data I'm adding on are the orders called  
20 intersex in these three laboratories. They are the green panels.  
21 Dupreez didn't record any. He looked for them, but didn't record any.

1           Hecker asked for them and didn't record any. Carr found some  
2           at the high dose only, but it is an alert. The reason that those two  
3           columns are not combined, the top dose in Carr, is that this  
4           potentiality for double-scoring.

5           So, one animal may have had both conditions. You can't add  
6           the two columns together. This is a problem of double-scoring. If  
7           that should become important, it probably can be back segregated to  
8           find out the number of double-scorers and then up individual events in  
9           animals.

10           And in the Hecker study, there was also this last terminology of  
11           mixed sex as opposed to intersex. Again, what all these terms mean  
12           there is the potential there, just show you in case you didn't see what  
13           happened, I brought up the potential for there being similarities in  
14           some of these terminologies and also in the intersex, mixed sex.

15           It is unclear whether a hermaphrodite may be related to  
16           intersex, may be related to mixed sex. So, in comparing the different  
17           studies, I think we're all a bit of a hostage to fortune because the  
18           terminology is so imprecise.

19           That's the end of the -- my analysis of the laevis Laboratory  
20           studies. That's PNAS and subsequent studies.

21           Now, fast on the heels of that paper came the EHP paper, which

1 is actually also very interesting from Hayes. This is laboratory  
2 studies in rana.

3 Here we have the terms, different terms to the ones that Hayes  
4 was using. Now the terms are dysgenesis and sex reversal. Those  
5 terms may be species specific terms or they may be just the choice of  
6 different terms or they may be the development of terminology. I  
7 don't know. But they are different terms.

8 Now, there is dose response and that is really interesting, those  
9 responses, the inverse, especially in these days of low-dose effects.  
10 Now, anybody who sees an inverse dose response for two doses, the  
11 first thing you must do is repeat the experiment.

12 Unfortunately, I'm sure you will know in the rather chaotic  
13 situation that's going on in low-dose endocrine disruption research,  
14 the first endocrine disruption is turning out to be -- publish the first  
15 experiment. Without exception all of the claimed low-dose effects  
16 where high-dose effects are absent or attenuated, the original  
17 observations are not repeated before publication. That is left for  
18 other people to do and then a row develops when the effect can't be  
19 seen.

20 I think we have a basic scientific problem here of dramatic  
21 effects not repeated before publication or if they are, are not included

1 in the publication. The more dramatic the effect the more work is  
2 required before publication in my view. Also, it is quite critical to  
3 have the base data and some knowledge of standard deviation,  
4 standard errors in individual animal data.

5 The claims are so great of low-dose -- the implications of the  
6 low-dose effect and inverted dose response is so great that you should  
7 provide all the data and clarify the situation at the moment of birth  
8 and it hasn't happened. So, although the effect is very interesting and  
9 very dramatic, I'm not sure what to do with it and we're not sure what  
10 to do with it.

11 One thing you do is try and repeat it. Hecker's study in John  
12 Giesy's lab attempted to repeat it. The terminology is their  
13 terminology. There is no effect at all.

14 We have the potential problem -- again, I'm just sweeping  
15 between changing terminology at the bottom there. It is not too clear  
16 what the terminologies mean. That's another problem with us all to  
17 the time.

18 There has only been that one repeat of this at the moment. So,  
19 this is an emotive claim and it requires further work. It certainly  
20 needs resolving; we need to know one way or to the other because if  
21 lower -- if the lower the dose the greater the effect, then all risk

1 assessment paradigms evaporate.

2       There is one more set of data which I'm going to show because I  
3 don't want to leave out data that may be considered inconvenient to  
4 my talk -- that's the Tavera-Mendoza data. It is rather dramatic. It's  
5 a change within 48 hours of the relative population of prime and  
6 secondary the appearance of atresia and changes in the volume and the  
7 number of nurse cells.

8       Now, I condensed it all into one slide. On the left are the  
9 permanent which applies to the first three columns, which is a  
10 reduction in prime and an increase in secondary, and an increase in  
11 atresia. Then the next two just revert to the volume of the testes and  
12 the number of cells.

13       Now again, this is quite a big claim and at this stage, it could  
14 turn into an hours's lecture. What I'm going to do is refer the panel to  
15 the comments made yesterday by Dr. Solomon, because his comments  
16 were actually quite provocative comments. He has written those up  
17 overnight and has made them into a white paper which will be given  
18 to the panel.

19       If you say things about the study you must be prepared to write  
20 them down and keep those written down and you will get those later  
21 on today.

1           Before I leave this slide, I think the biggest problem I have  
2 with it, obviously the fact that in the thesis there is a repeat study  
3 which was not positive, that's one problem, but at the biology level,  
4 that's a tremendous amount of restructuring to have gone on in 48  
5 hours. We just finished a genomics analysis of the uterotropic assay  
6 where we are checking what is going on in the uterus every few hours  
7 through the whole three days of the experiment.

8           By 48 hours, the process of remodeling is really just starting.  
9 You just started the wave of cell division. You are beginning to go  
10 make new cells and the uterus is starting to remodel. That's just so  
11 much biology to have occurred in 48 hours. It makes me worried. I  
12 think it is worthy of great scrutiny, this study.

13           Now, reproducibility is one of the real issues of science. I'm  
14 going to show you now just one way of looking at the overall level of  
15 reproducibility between the studies, the lab studies I have looked at.  
16 It is just a visual impression of the percentage effected, the dose of  
17 atrazine and the dose responses joined up as lines which you have  
18 seen as bar charts earlier. So, there's the original Hayes data.

19           I'm now just adding up all the Hecker and Carr repeats. This is  
20 on gonadal abnormalities. There is the whole data set. There is  
21 reproducibility problem there. On the intersex there is to the

31

1 original. In Hayes claim there is the dose response of Hayes and there  
2 is the several repeats we're trying to do.

3 This is -- whatever the reasons for this reproducibility, there is  
4 a problem of reproducibility. I just remind you of the NIH  
5 definition. I think we must stick to this primary criteria in the  
6 reproducibility replication is one of the most important things in  
7 science.

8 Obviously, listening to this panel and understanding these  
9 studies you could immediately come back and say, but people have  
10 done different things. They haven't repeated what I did. They  
11 changed the stage, they've done this, they've done that. While that is  
12 a problem, it's a problem of science. It is not actually a problem of  
13 this atrazine problem scenario.

14 I just bring one other quote to you from the best in the last 10  
15 years. It is a condensation of the whole fantastic life of Stephen Jay  
16 Gould. You must read the book. He makes this point. The  
17 replication with difference is one of the most important things we  
18 need.

19 We don't want replication of identical experiments. We want to  
20 see how firm, robust the observation is when you start making  
21 changes does it still hold up. While some of the changes you are

1 going consider may weaken some of the repeats and some of the  
2 original observations it is worth bearing in mind that difference is  
3 important when we're going to extrapolate across all amphibians and  
4 in all parts of this planet.

5 So, the conclusion for this part of the lab studies, I think these  
6 reported effects of atrazine on amphibian gonadal development are  
7 inconsistent between laboratories, no getting away from that. A  
8 subsidiary conclusion is that the major dramatic effects to date have  
9 not been confirmed.

10 There has been one study in Carr where a small effect was seen  
11 at the high-dose but the major effects are so far not being confirmed.  
12 Why that is must be one of the subjects that you consider.

13 At this stage, and I really feel strongly about this, there is a  
14 requirement for a confirmed effect that anybody in a competent  
15 laboratory can reproduce before you start worrying about mode of  
16 action.

17 The fact that I've spent the last six years looking at various  
18 people's low claims for low-dose endocrine disruption is with the  
19 single goal of trapping one of them in my laboratory so we can  
20 understand what is going on with toxico-gonadical analysis. We have  
21 been unable to, so it is critical to get the effect first.

1           Field studies. We are near to the end. This is the nature paper,  
2           rana pipiens. In fact, there is two papers. It appeared in the Hayes  
3           paper, it appeared in nature and it appeared very rapidly afterwards in  
4           DHP. It is essentially did same data -- there are differences between  
5           the publications, which are minor or typographical which I'm not  
6           going to go into.

7           These are the eight sites in America were studied across  
8           America. In all except site seven, atrazine was measured at the time  
9           of the collection of the frogs.

10          There is quite a range there; site six obviously has a lot of  
11          atrazine in it. The rest have just got some atrazine in it. Site 6 then  
12          becomes one of the really interesting sites.

13          The first thing is that the two sorts of abnormality referred to  
14          by Hayes in that paper -- there's dysgenesis and hermaphroditism.  
15          Dysgenesis occurred only at one site. It wasn't one of the sites which  
16          at the time had a high atrazine concentration. No other site were the  
17          dysgenesis. This word, dysgenesis, is interesting because it was used  
18          in the rana lab studies by Hayes. It is presumably the same effect  
19          being produced by atrazine in the lab studies.

20          Because its only in one site, I think you can pretty definitively  
21          say that those dysgenesis effects were not caused by atrazine. That's

1 just the scientific process of correlation.

2 The second parameter that was included was hermaphroditism  
3 and that's it here. In the rana laboratory studies of Hayes, he used the  
4 word, dysgenesis and sex reversal. Now we're having a dysgenesis  
5 and hermaphroditism which suggests that those two may be the same.  
6 I don't know. I stand to be corrected.

7 Now, my first site when I look at that is there is no correlation.  
8 It seems almost a reverse correlation between atrazine exposure and  
9 total gonadal abnormalities.

10 So, when you claim a correlation and it is not apparent, you  
11 have to find reasons for the exceptions and one of the exceptions that  
12 was mentioned in the nature paper was that the sampling time when  
13 the frogs were collected, it was the inappropriate time to be looking.

14 You should have been looking earlier on at the atrazine  
15 concentrations or the organogenesis was going on and the sex  
16 determination was going on. That's a two-edge sword.

17 For example, you can go to site six, where you have high  
18 atrazine and low abnormalities and say, well, they have been low  
19 atrazine levels at that time and then you can go to site three, which  
20 has got high low atrazine and high abnormalities and say, well,  
21 perhaps early in the year there was a real load of atrazine there. You

35

1 can do those sort of things, but unless you have got some data, you  
2 can speculate forever.

3 In that paper, Hayes suggests that the problem that he is facing  
4 in sites two and three, where there is a very marked difference in  
5 hermaphroditism time and identical levels of atrazine, the suggestion  
6 is that site two was only intermittent use and that site three it was  
7 sustained use of atrazine. I don't know data to support that.

8 There is another speculation in the nature paper that that very high  
9 abnormality level at site three was due to run-off from neighboring  
10 states because there aren't any farms in that area or wind transfer.

11 Again, that could be looked at, and the more you look at it the  
12 more problematic it gets in terms of water flow in the Wyoming area  
13 and the actual amounts of atrazine that might have to be carried in the  
14 air.

15 So, I'm worried about speculations when you haven't got a  
16 correlation when you are trying to make a correlation. Again, another  
17 problem with this is there are no base data in this paper. There are  
18 tables with means and arrows on them sometimes arrows on them.

19 And so the desire of a scientist, when he sees his data set,  
20 which is critically important to him and this is critically important to  
21 us, is to try and regenerate the data. I spent half my life now running

1 programs where you can put the end value a mean and an S mean and  
2 try and generate individual data and you can do it, but it's a lot of  
3 work. People tend to avoid that.

4 An endocrine disruption based-data is very, very, rarely shown.  
5 It is always means in standard deviations. When you try that trick  
6 with this one -- and I'm only going to give you one example, you hit  
7 problems. That is that in the method it says there are 20 frogs the  
8 collected from each site. That makes one frog equals five-percent.

9 So, then when you turn the page and you start reading about 92-  
10 percent of frogs effected or 28-percent of frogs effected and they are  
11 both direct quotes, you just don't know how that can be. Either frogs  
12 have died or frogs have been lost or slides have been lost or there is  
13 just no explanation and you can't get back to the base-data.

14 So, I think there this is another generic problem of data quality  
15 in endocrine disruption. It is not just this particular topic that seems  
16 to be endemic to endocrine disruption.

17 Now, soon after the publication of that field study, another  
18 field study came up, which certainly in the popular press seemed to  
19 me this is game-set and match the whole thing is adding up all over  
20 the world there are problems. That is the Cane Toad study, *Bufo*  
21 *marinus* of Tim Gross.

1           Now the Cane Toads in the field -- in the sugarcane fields and  
2           atrazine and many other chemicals used in those sugarcane fields -- it  
3           was a very bright place to go and look for effects. The Bufo has got  
4           these markings, the females below, the males above -- very  
5           distinctive.

6           Tim Gross looked at two fields and Christa McKoy looked at  
7           two fields. There are the numbers, originally about 50 in each of the  
8           fields and the controls for the University of Miami.

9           There were big effects; in the fields there were no male  
10          markings. Apparently every toad you picked up was a female.  
11          Amongst those that you picked up, 30 percent were hermaphrodite and  
12          there were no hermaphrodites in the controls from the University of  
13          Miami. These are big dramatic effects.

14          The first the world knew about this work was from post to the  
15          Christa and Tim gave and Rebecca rana from the Environmental  
16          Science and Technology, interviewed them.

17          In this article she wrote, which is the first we knew about it,  
18          she gave four quotes which is sort of interesting. The first one that  
19          these data suggests an external estrogen and they do suggest an  
20          external estrogen. Tim was aware, as we were aware, actually,  
21          atrazine is not an estrogen. These are direct quotes from that paper.

1           The next thing is the atrazine levels as in the Hayes study were  
2 unknown at the crucial metamorphosis period. Many atrazine  
3 measurements were made but not at the time of collection and during  
4 the time of collection. There was an admission that there are other  
5 chemicals present and there are many other chemicals present.

6           This quote here seems to be the eternal quotation for atrazine in  
7 frogs that the data raise more question than they answer. Our  
8 problems, when we try to bring these data in to decide what it means  
9 to us for our compound is that -- this bullet point I just added here --  
10 that the physical and chemical differences between the two sites have  
11 really not been assessed.

12           There are chemical differences between those fields, and the  
13 University of Miami and there probably are many physical  
14 differences. Just literally the physical difference between living in a  
15 chemically modified sugarcane field and being at University.

16           And some of those differences require to be looked at. Perhaps  
17 also the potential of stress differences and stress can effect the  
18 parameters we're looking at. There may have been different stress  
19 conditions in collecting frogs from the University pools and the  
20 fields.

21           Now you heard yesterday and updated these data and that's

39

1 another interesting thing. That is, these figures here in red, I hope  
2 you can see, after another year the previous one was spring of 2002.  
3 Now we're in spring of 2003. Red numbers are the current numbers.  
4 They have essentially doubled in all the groups.

5 Amongst the markings, the incidence has now dropped from one  
6 hundred percent to 76 percent. So, there are now being found some  
7 male markings. The Hermaphrodite percentage in the fields has  
8 dropped from 30 to 24 and the controls have now gone from 0 to 7-  
9 percent.

10 This is obviously a developing scenario. There is still some  
11 uncertainty there. I presume we're going to have 2004 data.

12 There are two other studies we'll just briefly look at you have  
13 in front of you. These are field studies conducted pursuant to these  
14 other field studies we have looked at. These are expressed as just  
15 total abnormalities. The first is one conducted by Smith, which is  
16 part of the DuPreez Group, which is in South Africa. You heard about  
17 that yesterday.

18 The atrazine measurements -- quite substantial differences --  
19 10-fold difference between the reference site and the agricultural site.  
20 These -- no effects observed there.

21 And likewise there has been one done at the University of

40

1 Michigan, a field study in juveniles and adults, with a much larger  
2 differential between the atrazine concentrations and there is no  
3 effects.

4 Now, one of the problems -- and I referred to this earlier and  
5 I've just illustrated this and I hope -- the panel must come to grips  
6 with it before -- I think, before you can make any decisions and that's  
7 just terminology.

8 Up here there is not a vast literature, actually, but this is what  
9 we found from the literature in a range of species. There is various  
10 rana species, and Bufo species and xenopus laevis, then there is the  
11 cricket frog, then there is the painted frog, and then there is the  
12 African tree frog and in field of green, laboratories, blue. In general,  
13 people are recording abnormalities.

14 So, this makes it interesting. I have made this red -- this here,  
15 because in all of Tyrone Hayes's publications there is never anything  
16 in the controls. In the xenopus laevis he now has no abnormalities  
17 amongst 10,000 controls and that's a vast control database.

18 In rana pipiens, zero incidence amongst 7,000. We heard  
19 yesterday from some of the members of the panel that they have not  
20 seen gross hermaphroditism in their controls. He have heard from Dr.  
21 DuPreez that he had not seen gross hermaphroditism. So, this raises

41

1 the issue of what people are calling abnormalities.

2 I really can't believe that all of those data to the left represent  
3 people defining things that are never ever seen by Tyrone Hayes. It  
4 has got to be terminological. I think it has got to be resolved before  
5 we can make progress on this. Conclusion 5. There is range of  
6 them that matter to us as we take care of the safety and the wildlife  
7 safety of the use of atrazine. The major effects reported by Hayes on  
8 gonadal development in the field were not confirmed in subsequent  
9 studies, the major effects.

10 The correlation between the level of exposure to atrazine and  
11 the observed effects is in the field studies remarkably weak. In fact, I  
12 don't actually think the word correlation applies. There is high  
13 variability between control abnormality levels which is probably not  
14 absolute. It is terminological. Other physical and chemical factors  
15 have not been adequately evaluated.

16 That's the end of my data analysis, but I want to end before I  
17 hand over to Charles Breckenridge with just a brief mention of this  
18 aromatase, because every newspaper or television program you read  
19 about or you hear about atrazine, aromatase comes into it.

20 As I said earlier on, we really do need to agree to fix -- before  
21 we study mechanism, but this aromatase is at 19, which takes and

1 testosterone into an estradiol, is the favored one.

2           And the most quoted data to support this -- and actually, the  
3 most relevant perhaps are those of Lou Guillette's Group. They did  
4 this study in '96. I showed you the sex reversal data. The absence of  
5 any fractures. Later on they took alligator eggs and they gave these  
6 three doses of atrazine. Now there are very high doses, they are  
7 expressed as part per million in the paper, but we have been talking  
8 micrograms per liter or parts per billion in this discussion.

9           So, that's actually 14 thousand parts per billion, which is very,  
10 very high, approaching the limits of solubility.

11           Lou painted his eggs with ethanol solution. In the first paper,  
12 which is usually the one that is quoted and it's not the best one to  
13 quote if you are trying to make a point actually, is the HP paper. He  
14 had male and female temperatures.

15           I'm only showing the male producing temperature, 33-degrees.  
16 This controls -- and this aromatase is measured in the gonadal  
17 anurans. The controls level are low in this mal-producing  
18 temperature and they are high. They are about four or five on that  
19 scale of females of 31-degrees.

20           Now, the estradiol and the estradiol and the tamoxifen were in  
21 this positive controls. The function of a positive control was to cause

1 total sex reversal, which they did. The function wasn't actually to be  
2 in a positive controls for aromatase induction. Estradiol should not  
3 induce aromatase and it doesn't because there is nonsignificant  
4 increase there, but it is increased. atrazine had this small increase,  
5 which was non significant.

6 There are three doses there, but the first statistical analysis  
7 that Lou did was to see if those played a role and those played no role  
8 in any of these experiments. So, dose was taken out of the initial  
9 Nova. That's why you have just got one bar for three dose levels -- a  
10 nonsignificant increase. Tamoxifen did cause a very clear induction.

11 I don't know why that is. Tamoxifen is a strange beast. It's an  
12 estrogen and an estrogen antagonist. So, it is a mixed agent used for  
13 breast cancer treatment.

14 Those data then were subsequently published in two --  
15 reanalyzed in two papers. One in a book chapter and one again in this  
16 pituitary article. Despite the absence of a dose correlation, they went  
17 back -- and which is a legitimate things to do and looked at the  
18 statistics for the individual doses.

19 When they did that, they found that the high dose gave a  
20 significant increase in aromatase. But there is one thing and that's  
21 about the dose, which I have shown you here. These are very high

44

1 doses.

2 All of the data we have heard about so far in the frog studies  
3 are on the left there at the light blue area and these alligator data are  
4 parts per million are up three orders of magnitude to the right.

5 So, if you do start invoking this alligator, which is sort of  
6 close -- close to a frog -- if you start invoking this data to support the  
7 mode of action, you have a very weak effect. The top dose and the top  
8 dose is about three orders of magnitude higher than anything we have  
9 ever had in frogs.

10 The two -- the three conclusions that matter from this study --  
11 both estradiol and tamoxifen produced 100-percent reversal of sex.  
12 Normal sex ratios were noted for atrazine, no effect at all in the sex  
13 ratio. That margin induction did not affect sex ratio.

14 Secondly, the estradiol testosterone and estradiol testosterone  
15 ratios were constant across all test groups. So, it doesn't sense to be a  
16 sensitive marker for induction of aromatase which I think we know  
17 from other studies. Lou Guillette does experiments well. He looked  
18 at testicular morphology and that was unaffected by atrazine.

19 So, that leads to my final conclusion, really, that the alligator  
20 aromatase data do not provide a firm foundation from which to build a  
21 mode of action.

1           My summary conclusions you have seen them all, so I'll just run  
2 through them. atrazine does not or alter sex ratio in reptiles or  
3 amphibians. Two studies have failed to reproduce the reported effects  
4 of atrazine on xenopus laryngeal size.

5           The major gonadal effects reported for atrazine in initial  
6 laboratory and field studies have not been confirmed with subsequent  
7 studies. Further studies required to resolve inconsistencies between  
8 the laboratory studies.

9           Inconsistencies also evident in field studies and should be  
10 examined if future laboratories studies warrant that. Further focused  
11 work required to produce a reproducible within lab and between lab,  
12 dose related effect before evaluating a mode of action.

13           Our recommendations are actually very similar to the draft  
14 recommendations of the EPA, because once you approach things  
15 scientifically, you come to the same conclusions. We need to focus  
16 on tiered approach to morphological gonadal endpoints and  
17 laboratories studies, I think, first, because you've got greater control  
18 over what you are doing.

19           Probably best to select xenopus laevis, because of it's data base  
20 with an atrazine dose response. If you start seeing things that you  
21 confirm to be real then extend rana so that you are moving towards

46

1 natural populations.

2 Quite critically there is a need to define conditions precisely,  
3 including husbandry, stage of development and the statistical power  
4 of your studies. Define gonadal terminology before you do anything.

5 Establish background incidence of the agreed gonadal terms in  
6 the control population before you start testing anything.

7 Conduct the studies -- I know it is very difficult and we don't  
8 have standard protocols yet in GLPs isn't everywhere. But it should  
9 be at least according to GLP, so there is a data trail and so that  
10 somebody can come in and see your data or you can make your  
11 primary data available to other people in a very user-friendly form,  
12 not one where you have to sit down for two days trying to describe it.

13 You need primary data and the ability to other people to come  
14 in and see your slides. Of course, if the effects are identified, study a  
15 mode of action and determine the functional significance of what you  
16 found.

17 Thank you for your attention.

18 DR. ROBERTS: Thank you, Dr. Ashby. If you are  
19 willing, I would like to offer the panel the opportunity to ask you any  
20 questions they might, have based on your presentation. I have a quick  
21 one and then I will let Dr. Kelley ask one.

1           On the Guillette studies, the alligator egg studies, are the  
2 concentrations of atrazine -- is that the concentration measured inside  
3 the egg or...

4           DR. ASHBY: No. No. It is an ethanol solution painted on the  
5 egg. I have been chitchatting on the email with Lou in the last few  
6 days, trying to get to grips with all of this. So, he knows -- he has no  
7 idea what gets into the egg.

8           The best guide he's got is when he does this sort of thing with  
9 the natural hormones like estradiol, about eight percent gets in. And  
10 so, you've really got to take one of those logs off of that scale I  
11 showed you there when are you doing the comparison. But those two  
12 comparisons are made about a log apart.

13          DR. ROBERTS: So, it is a complication in terms of trying to  
14 compare the concentrations?

15          DR. ASHBY: Yes, it is, but you still have one log in your  
16 favor. They would be touching each other if you apply the eight-  
17 percent, the alligator eggs would start where the field studies and lab  
18 studies have stopped.

19          Dr. Kelley.

20          DR. KELLEY: Just to follow up on the alligator. The alligator  
21 does develop, doesn't it, within a separate egg as opposed to the

1 amphibians, which hatch and are more closely to the medium.

2 DR. ASHBY: That's absolutely true, yes.

3 DR. KELLEY: So, without know it, we really can't compare the  
4 reptiles and amphibians very easily without knowing the effect of  
5 concentration of atrazine, would you agree?

6 DR. ASHBY: Absolutely. I only raised it up, because those  
7 Guillette data are the primary reference source. So, I mean, Tyrone  
8 always references that paper when he is talking about aromatase. It's  
9 the primary reference. Whether or not it should be, I agree with you.  
10 So, it is a different situation.

11 DR. KELLEY: So, I would like -- it is clear from your  
12 discussion in all of our discussions that we have to be very precise in  
13 our use of terms. So, I would like to address the issue of sex ratio.

14 In studies in xenopus, could you give me your definition of how  
15 a sex ratio should be determined and in particular, could you  
16 distinguish between genotypic sex and phenotypic sex.

17 DR. ASHBY: First of all I'm not an amphibian expert. So, I  
18 think that's a very valid question and I hope the SAP are going to  
19 approach it rather than me.

20 The reason is a particularly interesting question is that in  
21 readiness for this panel I have been reading the recent papers of Dr.

1 Claris (ph) and those have opened my mind totally.

2       Where we're talking -- where he is going with genotypic males  
3 and genotypic females, assessing the levels of testosterone maternal  
4 carry over and then talking about highly specifics, which is with five  
5 alpha reductase (ph) determining the phenotypic sex of what may not  
6 be the same genotypic sex.

7       So, I mean the expertise is on this panel. It is not my area. I  
8 have not done a frog experiment. So, there is no need to ask me.  
9 Some of my colleagues have worked with frogs. I don't think it's a  
10 productive question to ask us, actually, but it's a highly productive  
11 question to come to terms with amongst yourself as the SAP, I think.

12       DR. KELLEY: So, typically when you talk about a sex ratio  
13 you talk about the percentage of males and females and let me just  
14 point out that in xenopus the only real way to know if you have a  
15 genotypic male or a female is to back cross.

16       So, if you have an animal with ovaries, it could be genotypic  
17 male that had been feminized or it could be a genotypic female. If  
18 you then back cross that individual to a genotypic phenotypic male,  
19 which in this case is believed to be ZZ and look at the percentage of  
20 to the offspring, only then can you make an inference about sex ratio.

21       So, the only point that I want to bring here is that if you say

50

1 that you have not demonstrated an effect on sex ratio unless you  
2 actually know what the sex ratio is, you actually cannot draw that  
3 conclusion.

4 DR. ASHBY: That's right. So, I mean what we're actually  
5 saying is the original claim by gross in the turtles was badly formed  
6 in the context of what you are saying.

7 Your question is really addressed to all the people who have  
8 done all these experiments the phenotypic observation of what they  
9 call sex ratio was not confirmed in the original definitions and their  
10 ongoing definition is probably imprecise.

11 So, yes, I think we are defining further the insecurity of the  
12 experimentation.

13 DR. KELLEY: But you would agree, wouldn't you, that you  
14 could not draw a conclusion about sex ratio and the effect of atrazine  
15 on sex ratio without knowing what the sex ratio actually was?

16 DR. ASHBY: No, I wouldn't, because we have in the literature  
17 what experts are calling a sex change, a sex ratio change and that  
18 claim cannot be confirmed.

19 So, you may be correct, but it applies to everything. Nobody  
20 who has worked in this area can make any claims about sex ratio  
21 because they not been doing genotyping. The original claim that the

51

1 turtle had a sex ratio change has not been confirmed with other  
2 experiments at the same caliber with the same level of definition  
3 terms.

4 So, we're both -- I'm agreeing with you but, I think it is generic  
5 across the science, not just what I was saying.

6 DR. KELLEY: So, you are saying -- so, I think we're in  
7 agreement here, that they can't claim it and you can't claim it.

8 DR. ASHBY: Right.

9 DR. KELLEY: Thank you.

10 DR. ROBERTS: Dr. Green.

11 DR. ASHBY: But the problem is bigger than that because what  
12 you are saying is that everybody who talks about sex ratios in the  
13 literature is not talking science up until now, unless of course have  
14 you done some of these experiments. So -- and others like you must  
15 have done some.

16 But what is currently being called sex ratio in issues such as  
17 atrazine is an inappropriate use of terminology. All you can do is  
18 repeat phenomenology and not repeat it.

19 DR. KELLEY: You can just use the word, "Phenotypic" and get  
20 out of that bind. DR. ASHBY: Okay. Phenotypic changes were  
21 not confirmed in our studies, right.

1 DR. GREEN: I have a different question altogether. This  
2 primarily involves basic pharmacology and toxicological differences  
3 between reptiles, amphibians and mammals.

4 I think we would all agree that comparison of reptilian  
5 pharmacokinetics, for any compound to a mammalian would highlight  
6 some of the differences between those species. For example, we know  
7 certain metabolic pathways for biotransformation aren't present in  
8 reptiles and amphibians or at least present to the degree that they are  
9 in mammals.

10 So, a lot of the variability in these studies can be explained by  
11 intra and enter-species differences. When it comes to the anurans, we  
12 have an added level of complexity there in that their metabolic rate is  
13 determined primarily by ambient temperature.

14 So, there is another confounding variable that will affect the  
15 kinetics. I guess what strikes me when I look through all of these  
16 studies is that I don't see experiments designed to evaluate the  
17 traditional things that we look at from a pharmacological perspective  
18 such as volume of distribution, clearance, half-life or tissue levels in  
19 the frogs that could correlate or help us define exactly what  
20 absorption of atrazine could or could not have an effect.

21 I just want to get your opinion as a representative from

1 Syngenta, is there some technical difficulty with doing this, with  
2 measuring the tissue levels in the animals, so that we can get a better  
3 understanding of what exactly hangs around after exposure.

4 And I will let you speak in just a second, but one thing I'm  
5 concerned about with all of these water studies, where we're putting  
6 the chemical in the water, the frog will absorb it and then excrete and  
7 in the case of *xenopus laevis*, they'll sit in it for three or four days  
8 and reabsorb the active metabolites and the inactive metabolites and  
9 any of the degradable, some of which, I believe, are as toxic as the  
10 parent compound. So, those things will fluctuate overtime. So,  
11 monitoring the water levels of atrazine at the beginning of the  
12 experiment or knowing what you put in and then it seems like you  
13 want to know at the point of sacrifice of the animal what they were, as  
14 well as studying the tissues.

15 So, if you could explain to me perhaps or clarify why those kind  
16 of studies haven't been done by many of the groups that were  
17 supported by Syngenta? I assume there might be difficulties with the  
18 atrazine tissue level defects, but I don't know.

19 DR. ASHBY: Yes. There are two points and then I hand it over  
20 to Charles who has some other points to make.

21 The first is, I agree with what you are saying, but again, it's a

1 generic statement. The science in general is not doing this at the  
2 moment and they probably should be. When this got to the point  
3 recently, where it was going ahead from SAP we had a meeting with  
4 Mel Anderson and the process started of trying to understand this.

5 It certainly needs a lot of detailed studies and the fear is it maybe  
6 different for rana, it may be different of a microbism than a field, but  
7 the actual trying to get together of one of these modeling  
8 compartmentalizations models that Mel Anderson developed -- we  
9 started to do -- it was most relevant initially, we were starting a  
10 couple years ago to think about the GrNH mechanism and that's where  
11 we wanted to know exactly what gets in.

12 It is there. Again, another problem is delaying until you get  
13 something you know is real and reproducible, because at the moment  
14 we don't know which effect we're chasing and we don't know whether  
15 it is atrazine or metabolite.

16 When you know the effect you can start asking questions, is it  
17 atrazine or is it metabolite? And then you can start doing these  
18 highly complex and time-absorbing studies but to just be looking at  
19 what happens to atrazine and metabolites in a system, it would be very  
20 difficult.

21 I think it should be considered in the future, but it hasn't been

1 done at the moment and nobody ever, ever does it until the very end  
2 when they are trying to explain their effects in terms of metabolites or  
3 species differences. Is this due to the fact that the mouse has got  
4 more chromatization than the rat. But you have always got a defined  
5 object that you are chasing and I don't see it yet that we have a model  
6 with an effect where we can ask these questions.

7 DR. KELLEY: Sure, and it may be also quite relevant that at  
8 certain levels -- tissue levels known in xenopus laevis that are tested  
9 in the laboratory, we know that it has no effect. Perhaps that's the  
10 outcome, that you can have X amount of atrazine in gonadal tissues  
11 and do not produce Hermaphrodites. So, it is a negative result but an  
12 important negative result.

13 DR. ASHBY: Yes, I mean, the other very important point, of  
14 course, is the general practice of people to use organic solvents when  
15 they are not needed. So, a lot of this data is involving ethanol as a  
16 solvent. That's just is not needed, because atrazine is soluble enough  
17 in water up to 30 milligrams per liter.

18 So, there should be no need for this artificial breaking through  
19 of eggs with ethanol, because it confuses everything.

20 So, I think that is probably all we need to say.

21 DR. ROBERTS: Dr. LeBlanc and then Dr. Isom.

1 DR. LEBLANC: Mode of action is an important issue that we  
2 need to contend with and for that reason I would like to revisit the  
3 alligator comparison for clarification. When we look at these studies  
4 with tadpoles, the concentrations of atrazine used parts per billion,  
5 that is micrograms of atrazine per liter of test solution that the  
6 tadpoles are in and for the alligators, these eggs aren't in water.

7 So, I wondered what the parts per billion represented. Is it  
8 micrograms per kilogram of egg or is it...

9 DR. ASHBY: It must be the solution. It is literally the solution  
10 that Lou is making up in ethanol and painting on the eggs and he must  
11 know the volume he is putting on and assuming 100-percent  
12 absorption would give you those parts per million in the egg, but of  
13 course, it isn't 100-percent absorption, it's probably not even 10-  
14 percent. If it was estradiol, it would be 10-percent.

15 So, it is the total application to the egg and the total -- and the  
16 assumption of total absorption is the high dose.

17 DR. LEBLANC: So, it is micrograms per unit volume of --

18 DR. ASHBY: It must be.

19 DR. LEBLANC: I think it is to the egg. I'm not sure as well,  
20 but I think the take-home message is -- from the comparison is that  
21 the data suggests that aromatase is induced by atrazine. Bus this

1 issue of dose comparison is probably not valid because the units are  
2 different.

3 DR. ASHBY: Yes. There again with an effect which is not  
4 dose-related and yet is broken up into dose and it's only the high dose  
5 and it's only weak, that would be nice to repeat it before we build  
6 anything on it.

7 DR. LEBLANC: Agreed.

8 DR. ROBERTS: Dr. Isom.

9 DR. ISOM: In follow-up on the aromatase, it is appears from  
10 what you presented there is some problems with definitions of the  
11 reproductive effects. But in the case of aromatase, we have an  
12 enzyme that can be quantitated. But perhaps there are some problems  
13 with regards to that too, in definition.

14 How would you define induction of aromatase in these studies  
15 that you reviewed and made comments on and then made the  
16 conclusion that there does not appear to be an induction?

17 DR. ASHBY: One is that the measurements people make that  
18 have to do with enzyme and actually look at the conversion with the  
19 isolated enzyme. You can see how much is formed. It is the  
20 functional level of enzyme.

21 Probably the most relevant and often the inferential way of

1 doing this is to see testosterone go down and you assume testosterone  
2 is being used up, but that of course, ignores all feedback mechanisms  
3 which is an unwise thing to do or the estradiol levels must go up.

4 For the induction to mean anything, you need the product. You  
5 need more estradiol in the animal. The real important observation is  
6 that your estradiol levels go higher than they should be.

7 This is one of the complications, because you can make a  
8 measurement of an induction of an enzyme's ability to transform  
9 testosterone in vitro, but if you then go into the animals you have to  
10 show an effect of estradiol.

11 That is one of the big problems, because John Giesy, several  
12 years ago, suggested a very clever idea, if only it was true, that the  
13 uterographic activity of known ethanol, which we all agree is a  
14 neurotrophic in the rodent, was actually not due to its intrinsic  
15 estrogen -- the estrogen receptor in the uterus, but by its ability to  
16 regulate estradiol and make estradiol in those animals and it was the  
17 estradiol that was making the uterus grow.

18 So, I set aside about six months checking this out, because we  
19 had just done a load of uterographic assays. We found no evidence of  
20 induction of the enzyme in vitro and we didn't find any increased  
21 estradiol levels in the animals so, we had to conclude it was the

1 intrinsic estronicity.

2         So, the jump from measuring enzyme activities to functional  
3 production of estradiol is a big jump. I think the answer to your  
4 question really is to relate it to observations, you need an increase in  
5 measurable estradiol in the animal or else it doesn't really matter.  
6 But that's in a sea of feedback mechanism. So, it is horrendously  
7 complex.

8         DR. ISOM: It seems to me, when I look at enzymes and enzyme  
9 inductions or activities, we also are concerned about the message  
10 level. We're also concerned about the protein expression in the  
11 catalytic activity. All of those are important when you consider  
12 induction.

13         And then, lastly, what type of standardization of the tissues that  
14 are being sampled in the animal have occurred in these studies. Are  
15 we really sampling the enzyme activity from the same tissues and then  
16 the developmental effects on that -- that enzyme.

17         DR. ASHBY: Yes. I mean, your previous point is very valid  
18 and Joe mentioned it yesterday. We are just tripping right into the  
19 edge of toxigenics and quantitative RTCPR.

20         And so, the future is going to be done at message level and  
21 protein level and it will just get rid of a whole load of rubbish that's

60

1 knocking around. It will still require the second question to be highly  
2 specific about the tissue are you sampling.

3 Because, I mean, this whole area is so perverse in making a man  
4 you turn on aromatase in the brain to produce low estradiol, but in  
5 that developing male fetus, there is not going to be estradiol floating  
6 around the fetus. It is only in that part of the brain. Likewise, the  
7 production epidermis needs required estradiol expression of  
8 aromatase.

9 So, you -- the more can dissect the tissue you are talking about  
10 and the more you can have precise RTPCR-type technology, then we  
11 answer these questions. I agree we're playing with very crude  
12 terminology here and crude observations up to now.

13 DR. ROBERTS: Dr. Delorme.

14 DR. DELORME: I just wanted to make a comment on Dr.  
15 Green's question, regarding why concentrations aren't measured in  
16 tissues. I think there may be another reason.

17 Traditionally, when you are doing risk assessments, you are  
18 assessing based on concentrations in an exposure media. If you are to  
19 have tissue concentrations, you add a couple levels of complexity to  
20 the assessment.

21 One, what tissues are you going to measure it in and two, we're

61

1 then going to -- as risk assessors, we're then going to have to model,  
2 somehow using a PBPK-type model or something like that,  
3 concentrations in tissues when we do our risk assessments.

4 DR. GREEN: There are standard protocols for what tissues that  
5 you would look at. A good toxicologist and a veterinary pathologist  
6 can do that pretty routinely. So, we can discuss that between us, if  
7 you'd like.

8 DR. DELORME: But, traditionally, in risk assessment, what we  
9 have done is we have used waterborne concentrations for organisms  
10 that live in water. So, I mean, there usually --

11 DR. GREEN: I think at this point -- and I recognize that and  
12 that's a valid point. My concern was trying to help get a handle on the  
13 extreme variability due to the husbandry, mainly temperatures,  
14 species differences and variations in application.

15 And I think some of that might be explained by looking at --  
16 directly at the tissue that it would end up in.

17 DR. DELORME: Agreed.

18 DR. ROBERTS: Dr. Kelley.

19 DR. KELLEY: Could -- I agree with you, of course,  
20 completely, about replication. The first thing that we do in science is  
21 to replicate and the typical protocol in replication is to take whatever

1 it was that the original study did procedurally, and exactly duplicate  
2 it and see if we come out with that result.

3 And I just wondered if to your knowledge, in any of these  
4 published studies there was this kind of very stringent replication of  
5 the original Hayes study in xenopus?

6 DR. ASHBY: Dr. Hayes is going to talk about his own  
7 replication, his laboratory. All we have to run on is his published and  
8 there is no replication in that.

9 All I can say is that the studies sort of -- the studies were setup  
10 were intended to be a repeat under the conditions in which frogs were  
11 handled in that particular laboratory to actually go back and -- I  
12 mean, you see, another problem is and it is almost unique to endocrine  
13 disruptions, people don't say what they are doing in their papers.  
14 They just give you minimal data.

15 For example, people very, very rarely tell you what the diet of  
16 their animals is. Things like that, you just haven't got a clue. So, you  
17 use your own diet and then later on you can't repeat it and people  
18 come back and say, well, you used the wrong diet. You say, which  
19 one should I have used.

20 So, a lot of these things that are not -- we haven't probably done  
21 precise repeats. Probably the only way to do that is take some frogs

63

1 into Professor Hayes's lab and actually stand there do the experiment.  
2 You normally assume in toxicology that an effect which is real and  
3 significant will survive the many small changes that we don't think  
4 are important.

5 The fact that this is not surviving the many small changes that  
6 we don't think are important means that there is either a problem with  
7 the original observation or some of these changes are important and  
8 that's a challenge to find out.

9 DR. KELLEY: Could I raise a question?

10 On what basis do you decide that small changes aren't  
11 important?

12 You know, it is clear from this discussion that the standards for  
13 -- standards for regular husbandry of the frogs and so forth are not  
14 very well established. So, perhaps, we might agree that we actually  
15 don't know the conditions that might also affect outcomes in these  
16 studies.

17 DR. ASHBY: Exactly, and puts huge pressure at this stage on  
18 development of assays in frogs for the originating laboratory,  
19 especially with very major claims to check some of these things  
20 themselves and actually repeat the experiment with a few changes.

21 You know, let's not be quite so fussy about that stage, let's

1 change the protocol. You know just check the heart at the -- the  
2 sturdiness of the observation. It's a matter of whose responsibility it  
3 is.

4 DR. KELLEY: Well, let me ask you a question.

5 Suppose somebody got a detailed protocol from Dr. Hayes, of  
6 how he raised his animals and so forth. It might be necessary to get  
7 water from Berkeley, but we could probably manage that and repeated  
8 the experiment and was not able to replicate the result, would you  
9 agree we could all go home? DR. ASHBY: No. I would then  
10 say what you have to do and I have lived through this 15 times in the  
11 last five years, you have to then start talking very seriously with the  
12 originating author and you have to get your heads together.

13 I have done this several times in the spirit of cooperation, not a  
14 confrontation. And you have to say, I have done as much as I come. I  
15 have a just done a study which is coming out in the next issue of  
16 Toxicological Pathology, where we did six experiments to try to  
17 repeat low dose back to PPA that had been reported in Japan in the  
18 rat.

19 The very last thing -- I went through three diets, because we  
20 thought diet might be the important thing, because there was a diet  
21 you can only buy in Japan and I got -- my third diet was as near as you

65

1 can get. In the end, talking to the Saree (ph) in Japan, we eventually  
2 shipped the diet from Japan, which is not available in England -- it  
3 cost me \$25,000 -- the diet cost \$200, getting it to England cost  
4 \$25,000 and we did the experiment again using his diet and there is  
5 still a difference.

6 So, we just said, we don't know what is going on. He is going  
7 to go back and do his experiment again and I might do mine. So,  
8 someone, some experiments however precise you get, you still don't  
9 get an outcome. You just -- this is life at the moment, especially with  
10 endocrine disruption.

11 DR. KELLEY: But to return to frogs just for a second, you  
12 would agree there has not been strict replication of the original study?

13 DR. ASHBY: I think that's probably true, yes.

14 DR. KELLEY: Thank you.

15 DR. ROBERTS: Thank you, Dr. Ashby. Let's move  
16 onto Dr. Breckenridge's presentation.

17 DR. BRECKENRIDGE: Good morning ladies and gentlemen. I  
18 am Charles Breckenridge. I'm a Senior Research Fellow. I've been  
19 working for about 15 to 17 years on the mammalian toxicity of  
20 atrazine and the mode of action underlying it's endocrine effects on  
21 mammalian systems. That is not going to an easy topic of my

1 discussion today.

2 I am more narrowly focusing on the question put forward as a  
3 plausible hypothesis to count for gonadal abnormalities that have  
4 been observed in xenopus and some models that have been used to  
5 evaluate those chemical induced sex reversals.

6 I would like to put in it the context of a little bit about what we  
7 know about atrazine's action.

8 I can also take any questions from the panel that you might  
9 have relating to the mammalian mechanisms because we heard some  
10 questions yesterday about that.

11 First starting point that the -- an uncertainty associated with the  
12 data sets that Dr. Giesy or John Ashby has discussed, suggests that  
13 we're a little bit premature to talk about mode of action discusses  
14 until we have some reliable phenomenon we can reproduce in the  
15 laboratory environment.

16 Nevertheless, the one topic that has come up repeatedly in  
17 papers and discussions is that atrazine is operating through an  
18 up-regulation of aromatase. Estrogenicity is being generally put  
19 aside. And because these questions about this particular proposed  
20 mode of action are present, we would like to make a few comments on  
21 that.

1           Before we do that though, I would like to go back to some basic  
2           descriptive work. This is not my area of expertise, but I find it very  
3           interesting and some of the new methodologies that are available can  
4           be applied to the developmental process that occurs in frogs during  
5           the critical period of sex differentiation.

6           In this slide, I'm reproducing results of information from  
7           Iwasawa. In this particular study, he looked at the developing larvae  
8           at stages of development, prior to metamorphose and made a  
9           determination of sex based on morphologic characteristics and  
10          observed, as would be expected, that there is -- the gonads are  
11          undifferentiated up to a point in time and beginning at about stage 51  
12          differentiation begins and it goes through completion, at least in his  
13          hands, in a short window of time ending around about 56 or 57.

14          This particular graph represents the proportion of animals that  
15          have reached that stage of determinations of whether the gonad is  
16          ovarian or testes in character. So, it is a morphologic  
17          characterization of a group of animals at different stages.

18          He had done a lot of work on this topic and certainly to the  
19          aspect of the role of estrogen and in "sex reversal," whether it is  
20          phenotypic or genotypic had been well described in the past and  
21          certainly that window and at least as defined by which he was

1 approximately from stage 51 through end of -- stage 55. And he  
2 established in those studies as well, that the dose of estradiol that  
3 would cause that sex reversal to be about 50 part per billion or 10 to  
4 the minus 7 more.

5 That's an interesting observation in it's own right in so far as 10  
6 to minus 7 more is about two orders of magnitude or three orders of  
7 magnitude lower than the KD for the estrogen receptor. So, the  
8 inference may be that perhaps this isn't operating through the normal  
9 processes in terms of affecting this differentiation.

10 In addition to that description by Witschi and Chang, there  
11 were other experiments -- it was recognized that the gonads seem to  
12 differentiate from rostral to caudal. There were about 14 groupings or  
13 clusters of cells that sequentially differentiated as time went on, in  
14 which he did a very elegant experiment to look at the time sequence  
15 of estradiol exposure -- two days of estradiol exposure applied during  
16 this critical period.

17 This particular representation I'm making now is sort of as you  
18 move from the left side to the right side you can regard it from rostral  
19 to caudal. He applied estradiol in two-day periods. He observed kind  
20 of a wave of feminization of the males extend down. And the  
21 presumption was that if you miss some of these periods of time, you

1 would perhaps get this intersex description. That is to say you would  
2 have partially transformed gonads and partially untransformed.

3 That was kind of an interesting observation in the light of some  
4 of the discussions we been having the last couple days about mixed  
5 sex, intersex, hermaphroditism and the appearance of the gonad.

6 Obviously, this is an estrogen mediated phenomenon. That's  
7 not necessarily what we're talking about here although the aromatase  
8 hypothesis implies that it is an estrogen mediated phenomenon that  
9 we're discussing with respect to atrazine exposure.

10 I also have to point out that even in the current literature,  
11 others have published things and you will find, in fact, in your white  
12 paper provided by EPA the suggestion that the critical period is  
13 earlier in time. I think this is figure 3. One needs to carefully  
14 look at all of these studies. This one is inconsistent with the rest of  
15 the literature as far as I can tell and principally, it shows the problem  
16 of the duration it takes for metamorphosis to occur and the impact of  
17 that perhaps. So, the whole -- even within the basic descriptive  
18 biology we're seeing some inconsistencies.

19 There is another factor relative to sex reversal by estrogen and  
20 that is it is not ubiquitously the same across all the species and I'm  
21 sure that many of you know this better than I do, this is just from an

70

1 extract of a paper by Wallace.

2 And again, it points to the fact that when we're talking about a  
3 surrogate for an native species Anuran, we have to think very  
4 carefully about what it means in regard to a compound induced effect  
5 in relationship to gonadal abnormalities.

6 So, in summary, there is some discussion yesterday about the  
7 critical dose of estradiol and why we were getting less than maximal  
8 effects in one hundred part better bill in some of these studies when in  
9 the literature, people are claiming that doses as low as 50 should be  
10 effective for 100-percent reversal. I make the point again about the  
11 estrogen receptor relative to these kinds of concentrations and where  
12 the receptors normally expecting to see the hormone regulated at.

13 We also note that if you move a couple of orders of magnitude  
14 lower, estradiol becomes toxic and creates malformations and death in  
15 the developing fetus.

16 So, we're talking about a phenomenon that has a -- which is, I  
17 think, not well understood even today in regard to estrogen induced  
18 sex reversal in the primary model we have being considered here.

19 The critical window of sensitivity is important and the  
20 importance of standardizing the rates of metamorphosis of these  
21 studies are to the time period in which metamorphosis occurs by

71

1 means of the quality of the culture methods obviously, is important if  
2 we're going to be comparing these things to each other. And then we  
3 note that there is the species differences in terms of sensitivity to this  
4 model.

5 In some of the newer technologies that exist and I'm going to  
6 overlay this information on the development of -- and I have chosen  
7 over here because this was the only place where I could find all of the  
8 critical data, so this is the graph that I had previously for females  
9 only.

10 This is from the paper by Iwasawa, again. He is classifying  
11 these animals as males or females. These are the ones that are female.  
12 He is displaying that along the developmental stage. I jump to a  
13 second paper, Miyashita, to get information about to the expression of  
14 the message for the estrogen receptor and I overlay that on the same  
15 time line relative to the gonadal differentiation.

16 And I also then from that same paper extract the message  
17 information for the zip 19 and overlay that. And from a third paper,  
18 look at the sort of the morphologic character of well, when are the  
19 follicles forming and when might that be considered to be perhaps a  
20 functional unit to be able to manufacture and secrete estrogen and  
21 convert testosterone.

1 I suggest to you that there perhaps methodologies in this  
2 critical window that allows us to get a better description of processes  
3 associated with differentiation and then the impact of a Xenobiotic on  
4 those processes.

5 How estrogen is involved in inducing or initiating sex reversal I  
6 don't think is being worked out thoroughly up to now and you are the  
7 research community that probably knows the strategies that could be  
8 used to augment that. But I think it is a key difficulty associated with  
9 this area.

10 So, just to turn back to atrazine and relative to direct  
11 estrogenicity as Dr. Ashby had indicated, the studies are generally  
12 negative across the entire spectrum. So, that atrazine is working  
13 directly as an estrogen in the sex reversal process and feminizing  
14 male anurans doesn't seem to be a plausible alternative.

15 The idea that atrazine might up regulate aromatase and thereby  
16 accomplish the same thing by delivering endogenous estrogen to that  
17 tissue and therefore feminize those males or partially feminize those  
18 males where you get some partial expression or conversion,  
19 phenotypically is not entirely supported by the data that we have up to  
20 now. That is the two studies that were referenced yesterday in regard  
21 to aromatase measures and evidence that they were altered in vivo

1 systems is not present.

2 We all recognize the limitations of those kind of experiments,  
3 but to the extent they have been evaluated at environmental or  
4 relevant concentrations, no change in aromatase had been observed.

5 And we had this morning ago little discussion on is the enzyme  
6 inducible. When I first started thinking about this problem relative  
7 to, you know, immediately say it's a P-450 enzyme and that is true but  
8 it is not an inducible enzyme like some of the mono-oxygenases that  
9 are there specifically for detoxification.

10 The range of induction in quotes is nowhere in the range that  
11 you expect for some of the P-450 enzymes like the phenobarbital-type  
12 inducers can change the expression of P-450 enzyme over several  
13 orders of magnitude, whereas the range of aromatase would say  
14 expression maybe a twofold, threefold, fourfold and one might regard  
15 that as within the context of the object of those enzymes is to convert  
16 testosterone locally to provide a delivered dose of estradiol.

17 If we're talking about DHT, the same kind of thing. It is a local  
18 control mechanism where there is entire pituitary hypothalamic access  
19 to sort of grossly regulate the hormone in the -- available to those  
20 tissues.

21 So, we discriminate then between induction versus expression

1 at least in the way I'm thinking about it and we note that in invitro  
2 studies where atrazine has been shown to increase aromatase in that  
3 Sanderson paper, which we'll describe briefly and the paper that Dr.  
4 Ashby mentioned earlier relative to turtle eggs.

5 Sanderson's model was to take a certain cell types, cell lines  
6 and put them in anginous media and add atrazine to that medium,  
7 concentrations raging from lower levels on up to -- near the solubility  
8 limit. He went up to 30 micro molar or 65 hundred part per billion.  
9 In two different cell lines the adrenal cortical cell and the placental  
10 cancer sell JEG-3, he observed a maximal two to two and a half folds  
11 increase in aromatase over that especially at the higher dose ranges.

12 At the lower levels, he saw no effect -- 73 part per billion in  
13 that solution was a no effect level for aromatase induction. In regard  
14 to other cell lines, the MCF7 he saw no effect at all. And I would  
15 jump down to the fourth line on that graph where he had carp treated  
16 in vivo and took the livers out and looked for evidence that  
17 vitellogenic couldn't be inducted by atrazine or blocking estrogen's  
18 ability to induce. That was rather a -- that was more indirect method  
19 or measure of atrazine's effect.

20 And then I finally will turn to the Spiteri study. These are the  
21 data that Dr. Ashby was showing with the left panel being the

1 information that he had presented for the gonadal aromatase.

2           And you will observe in the atrazine treated groups on the third  
3 grouping there of data in the left panel, that there is this apparent  
4 increase in aromatase at 14 thousand ppb and this was a nominal  
5 concentration. The authors painted the atrazine on the eggs with  
6 ethanol. They weighed the eggs and they determined the  
7 concentration as a nominal concentration in the egg.

8           What is interesting about this aromatase increase that is being  
9 reported here is there was no impact on "sex reversal." This is no  
10 phenotypic conversion of those males back to females, whereas 14  
11 part per billion put to the egg in the same kind of a model did 100  
12 percent conversion of those temperature dependent.

13           So, whatever that aromatase expression is reflecting at that  
14 concentrate -- in that particular model, it is not having a biologic  
15 consequence of to the same way that estradiol did. So, the  
16 endogenous estrogen production probably had to be below that level  
17 of the applied estradiol.

18           So overall, in regard to this particular mode of action, and I do  
19 want to say we recognize that Dr. Kloas has been doing some of this  
20 work also with the androgen receptor and the expression of it. We  
21 were looking to find information on the compliment of information,

1 such as I showed for the estrogen receptor. I wasn't able to piecemeal  
2 all of that together. So, we didn't use that example. It would have  
3 been as equally valid to look at antigen receptor expression and the  
4 DHT message and so on.

5 Those kinds of experiments would be instructive. In any case,  
6 as far as aromatase goes, we don't see any evidence that aromatase is  
7 up regulated invitro. There are some data invitro that suggest it can  
8 be. We note that in some of those studies there doesn't seem to be a  
9 linkage to consequences as far as the animal. And when do you see a  
10 change in aromatase with an unknown amount of applied atrazine to  
11 the egg or at least to the delivered dose of the atrazine to the inside of  
12 the egg is not known.

13 In regard to the basic model of changing in gonadal character  
14 phenotypic expression, we think that there still is enough uncertainty  
15 around that model at least as it appears in the literature, people who  
16 are reporting rather dramatic periods of differential in the critical  
17 window. The timing of the key events relative to the expression of  
18 the estrogen receptor and the aromatase enzyme or even the enzyme  
19 that converts testosterone to DHT.

20 Just the conceptual framework of understanding how an event  
21 that happens later in time can be involved in the process of the

1 induction of the sexual differentiation is interesting. The dose  
2 response characterization relative to estradiol or for having a theory  
3 that it is endogenous as to being delivered and doing the same thing.

4 One needs to get that in order and in line. The role of estrogen  
5 in -- the estrogen receptor expression relative to this process, is it  
6 independent of the expression of the estrogen receptor that we get the  
7 sex reversal, the role of aromatase expression and differences in  
8 species.

9 So, overall, I think some basic biology needs to be done when  
10 we're focusing on a model that talks about gonadal abnormalities and  
11 changes in gonadal abnormalities relative to a exogenous chemical  
12 that is put into the system, especially when we're invoking  
13 mechanisms that are through the pathways that have been well  
14 studied, that is estrogen induced sex reversal.

15 Overall, there is lack of consistent evidence to suggest that this  
16 particular hypothesis is accounting for the phenomenon which have  
17 many inconsistencies in their own right and we suggest that in all  
18 cases, this call for additional research is probably the prudent and  
19 necessary thing to try to do to try to clarify these matters. So, I leave  
20 off with there. I would be willing to answer any questions.

21 Thank you.

1 DR. ROBERTS: Thank you Dr. Breckenridge.

2 I believe Dr. Denver has a question for you.

3 DR. DENVER: A point of clarification, a question and a  
4 comment.

5 You mentioned that Sanderson's study showed a two and a half  
6 fold increase in aromatase, but I believe that was in the messenger R  
7 and A. So, perhaps a reflex of transcription. I think they also  
8 reported a 4- to 5-fold increase in the activity of the enzyme. I think  
9 it is important to first of all differentiate between the actual activity  
10 that you are measuring and also the transcription of the gene.

11 DR. BRECKENRIDGE: Yes, I believe you are totally correct in  
12 that clarification.

13 DR. DENVER: The other question I had was, has Syngenta  
14 attempted to repeat those studies of Sanderson?

15 DR. BRECKENRIDGE: We have not attempted to repeat them.  
16 They appear to be substantial and clear. That is to say the DOS  
17 response characters were seem to be pretty reliable and strong. We  
18 did note some inconsistencies. There was one of the metabolites of  
19 atrazine was tested, dymachloratrizne.

20 Intuitively there would be no reason to believe it wouldn't  
21 operate in the same way, yet it failed to have the effect that atrazine

1 or simazine or propiomazine (ph) which are members of the same  
2 class. We noted in that same study, hydroxy atrazine, where the  
3 chlorine is removed from the molecule, did not alter the expression of  
4 the message or aromatase levels and that would be consistent with our  
5 understanding of perhaps a structure basis for it.

6 We also in our discussions with our endocrine people recognize  
7 that estrogen and aromatase expression -- one can regard Dr. James  
8 Simpkins, who is our endocrine -- the leader of our human endocrine  
9 panel -- has a model for evaluating the beneficial effects of estrogen  
10 on -- the protective effects of estrogen on cells and basically it is an  
11 ischemia model where he causes cellular damage in the brain and the  
12 net result of that physical damage to cells is an up-regulation of  
13 aromatase and expression of estrogen. He fundamentally believes  
14 there is a certain amount of protective effects at least in the brain in  
15 regard to an aromatase expression.

16 We are mindful of that in the context of the cellular studies.  
17 We're not necessarily claiming those, that's the purpose or the reason  
18 why these cells are responding. That is, no cytotoxic or damage to  
19 these cells, but it's a possibility.

20 DR. DENVER: Well, assuming it is not a cytotoxic effect and  
21 granted that these are transformed cells and may not reflect the invivo

80

1 situation, has anyone considered using these cells, perhaps as a  
2 benchmark for aromatase induction by atrazine in any of the studies  
3 that have been conducted -- any of the amphibian studies or even  
4 mammalian studies?

5       Could these cells perhaps be valuable as a bioassay for assaying  
6 for the bioactivity of the atrazine in preparations made by different  
7 laboratories or simply validating the aromatase assays and showing  
8 the induction by atrazine.

9       DR. BRECKENRIDGE: As a bioassay for the presence of  
10 atrazine across labs, that -- I mean, obviously, the analytic message  
11 would be preferred just to quantify that.

12       DR. DENVER: The reason I bring that up is there is a lot of  
13 discussion of variance in the results obtained with different doses of  
14 atrazine and also potential vehicle effects, vehicle interactions with  
15 atrazine.

16       I wonder if some of these could be addressed using a cell based  
17 assay, which would be relatively inexpensive? The cells could  
18 potentially be cultured in the different laboratories and tested with  
19 the preparations that were going to be used in the amphibian assays or  
20 the preparations of atrazine could be sent to a central testing lab  
21 where they could be validated using a cell-based assay of this sort.

1 DR. BRECKENRIDGE: I guess, really, to properly answer that  
2 question in terms of ease of that kind of a program, maybe it would be  
3 better if Dr. Giesy addressed how reliable and uniform are those  
4 results in replicate. That would be a critical feature of a bioassay of  
5 that sort. I would have to defer to Giesy and Sanderson.

6 If you would like, Mr. Chairman, he could probably come up  
7 and answer that question.

8 The second phase of it though, I didn't understand -- if you  
9 could. You had a second question as part of that two-phase thing?

10 As a surrogate -- I think you were using these cells as a  
11 surrogate for the aromatase up-regulation in amphibians and I didn't  
12 quite understand what you are looking at.

13 DR. DENVER: No, I didn't intend to use it as a surrogate, but  
14 rather, I mean, they would be valuable for a number of reasons as a  
15 positive control for actually showing that the atrazine that was being  
16 used in the laboratory actually had some effect. That's what I'm  
17 referring to.

18 DR. BRECKENRIDGE: Thank you.

19 Mr. Chairman, would you like Dr. Giesy to come forward or do  
20 you want to defer that?

21 DR. ROBERTS: I have a number of other panel members lined

1 up for questions, but if Dr. Giesy could perhaps consult with Dr.  
2 Denver during a break and go over that.

3 Dr. Isom and next and Dr. Skelly and Dr. Kloas.

4 DR. ISOM: Thank you.

5 Dr. Denver touched on the first point or question I have, but if I  
6 recall correctly the Sanderson study was done in human tumor lines,  
7 mammalian cells and the question I would have, is there a difference  
8 in species effects on induction aromatase and are you aware of any  
9 studies that have been done across species on the enzyme?

10 DR. BRECKENRIDGE: I'm not aware of those studies as it  
11 relates to atrazine. DR. ISOM: It seems that the direction that  
12 Dr. Denver was going is that perhaps a study should be done in  
13 reptilian cells or cell cultures as opposed to humans, which the human  
14 cell lines could be used as perhaps a starting point and give you some  
15 dose response studies. But it appears that the -- really the end result  
16 should be done in appropriate specie's cells.

17 DR. BRECKENRIDGE: Yes. My view on invitro versus invivo  
18 obviously, is you go to the model system that is particularly relevant  
19 to the species and to the impact on that species. We're never sure  
20 what we're looking at when we're putting high concentrations directly  
21 with cells in invitro model. It is indicator of some potential

1 possibility of induction, but I don't think it implies necessarily  
2 within the whole animal that that would actually recur.

3 I would caution with having too much exuberance about the  
4 prospects of having that particular model being relevant to the whole  
5 animal.

6 DR. ISOM: A second question would be: Are you aware of any  
7 studies that have been done where a positive control inducer -- non-  
8 atrazine inducer of aromatase has been studied and it produces  
9 induction aromatase in the species and then secondly, we see changes  
10 in developmental effects?

11 DR. BRECKENRIDGE: We looked long and hard for inducer of  
12 aromatase in the literature.

13 The only thing we have come up with was plastic laden, perhaps  
14 and I think the positive control that Dr. Giesy used in his study was  
15 AMP. So, those are the only two agents that I'm aware of that -- there  
16 is a paper by Harris that talks about a plastic line in two and the  
17 positive control that Giesy used within this model. I'm not aware  
18 Xenobiotic that actually helps regulate aromatase.

19 DR. ISOM: if I recall correctly, I think Sanderson has used  
20 some fungicides in his paper to induce aromatase activities through  
21 inhibition of phosphodiesterase. To me, it seems the logical way to

1 go is be able to even -- to show that aromatase induction induces the  
2 biological effect that we're looking for here.

3 DR. BRECKENRIDGE: I find it interesting the concept of  
4 positive control. Is atrazine a positive control now for aromatase  
5 induction? I mean, when does it and how does an agent actually  
6 become the standard for a particular modality? I know there are  
7 studies on aromatase inhibitors and they describe them as  
8 nonestrogenic aromatase inhibitors.

9 How do you actually reach the viewpoint that perhaps there  
10 isn't an -- through an endocrine mechanism that those inhibitors are  
11 operating. So, one has to be almost -- it's a scientific consensus as to  
12 what constitutes positive control for a particular mode of action and  
13 we're talking about a mode of action that is aromatase up-regulation,  
14 which from a biologic perspective, xenobiotics aren't -- or the biology  
15 isn't designed to react to xenobiotics for up-regulation whereas, in the  
16 P450 enzymes, those enzymes there are specifically for the purpose to  
17 recognize the antibiotic.

18 I don't know what to say about the positive control dilemma  
19 associated with the aromatase part of this experiment.

20 DR. ROBERTS: Dr. Skelly.

21 DR. SKELLY: I will leave it to you to decide, Drs. Ashby and

1 Breckenridge, who is best able to answer my question, but based on  
2 both of your presentations, I caught a couple of themes. One is that  
3 the results that we're focusing on here, I've have shown very strong  
4 context dependence across studies within last labs and between labs.  
5 The second is that it may be premature to look for mode of action.  
6 The suggestion by Dr. Ashby that maybe the way to go -- the priority  
7 should be to start working on lab studies focused on xenopus laevis  
8 and looking at gonadal deformities.

9 I wondered what the rational is for putting field studies at a  
10 lower priority and at what point should the decision be made to do  
11 field studies and how would those be used is say, weight-of-evidence  
12 approach?

13 DR. ASHBY: The simple answer is that you can control  
14 conditions much better in a laboratory. I mean, perhaps up to now the  
15 control -- the conditions aren't being controlled precisely enough as  
16 was raised by earlier questions about whether or not they are exact  
17 repeats. But at least you know what is in the water. I mean, I think  
18 one of the main problems in field studies is what else is around. So,  
19 for example in the Crain study there are 13 chemicals. If you  
20 bothered to start looking into that and then you have to start asking, if  
21 they have played a part, what are they?

1           It is just a control that you know you are talking about a  
2 specific chemical, that's all.

3           I know -- I don't know there is not enough data yet, but you  
4 would not expect to find things that you see in the field that you don't  
5 see in the laboratory if it is a simple chemically media to do defect.  
6 You wouldn't expect interaction with some environmental condition,  
7 which only made it active in the field.

8           So, theoretically, you should be able to model this successfully  
9 in the laboratory and then get some knowledge of dose response and  
10 perhaps mode of action and then extrapolate into through to the field.

11           It just seems procedural, really, you can brings frogs in much  
12 easier than go out into the fields.

13           DR. SKELLY: I guess, as my follow-up, I've done a lot of  
14 experiments on amphibian larvae, both in the lab and field. There are  
15 things that involve interactions -- you know, synergisms between  
16 actors that you absolutely can't see in the lab that you can see in the  
17 field.

18           I guess I'm concerned that you might really miss something if  
19 there is some sort of synergism. And if the position -- if your  
20 position is that it is premature to consider mode of action, that sends  
21 me out to the field first. Maybe not first but at least simultaneously.

1 DR. ROBERTS: Dr. Kloas.

2 DR. KLOAS: I would like to give a comments about your  
3 presentation on estradiol use. First of all, you mentioned that there is  
4 a KD for the estrogen receptor of about  $10^{-10}$  molar. So, I'm  
5 only for available for one paper. I'm also a coauthor and we had  $10^{-10}$   
6 minus eight molar for xenopus.

7 DR. BRECKENRIDGE: Thank you very much for that question.

8  
9 I actually was taking that from my knowledge of the human  
10 estrogen receptor binding concept, so I didn't -- I was not able to find  
11 one for the amphibian and I appreciate that.

12 DR. KLOAS: It's Luetz and Lewis (ph) in 1999.

13 Secondly, for the critical dose things are also many reports in  
14 the past literature but also a more recent one, you can induce -- not in  
15 just male developing xenopus, but you can induce feminization in  
16 phenotypic getting more phenotypic of females for being in  
17 concordance with the terminology. At  $10^{-8}$  molar and  $10^{-9}$   
18 minus 9 molar it seems to be at least the tendency for getting  
19 feminization.

20 I think the doses you are dealing with -- the differences with  
21 what may happen with amphibian estrogen receptors is not so far

1 related and I would like to clarify it more.

2       Secondly, I think aromatase experiments *invivo* and using *x*  
3 *vivo* measurements of aromatase, personally, I think, as I mentioned  
4 yesterday also, then if you want to show up something, you need  
5 short-term exposures not long-term exposures. Then you have  
6 endocrine counter regulations.

7       We already discussed that yesterday, do it's not really, I think it  
8 is a good prove long-term experiments and having no differences and  
9 as we know it is a very -- rather difficult to assay aromatase and  
10 standard deviations, I think I would really rely on short-term  
11 exposures.

12       DR. BRECKENRIDGE: Thank you very much for those  
13 comments.

14       I put the challenges out more as a stimulus for thought, because  
15 I'm not an expert in this area. I have been reviewing that literature  
16 and looking at inconsistencies even within the literature and also  
17 recognizing some of these new methodologies can give a better  
18 resolution of time relative to dose and effect.

19       So, that's kind of -- the point I was hoping to stimulate this  
20 panel and others should think about because I think you get work  
21 locked into a paradigm and you sometimes look beyond maybe some

89

1 outside input as to other ways of looking at that paradigm.

2 DR. ROBERTS: Dr. Kelley.

3 DR. KELLEY: Could we turn to your second slide if you could  
4 switch to that?

5 This is the critical window side -- Critical window for estradiol  
6 induced sex reversal.

7 I would like to clarify the lapondo (ph) in Lario's (ph) results,  
8 because I see that they are confusing and hard to interpret. I see they  
9 have shown up, I think also in an inaccurate way in to the white paper  
10 from the EPA.

11 So, one of the points you wanted to make in this presentation as  
12 I understand it is that the literature is somewhat inconsistent in terms  
13 of what the critical window is for sex reversal and at what stages it  
14 occurs.

15 Let me just point out that in this Villapandos study, what they  
16 did was to begin treatment stage 44 with 100 micrograms per liter of  
17 E2 and they continued treatment for 90 days at which point their  
18 animals were somewhere between stage 56, which is the first  
19 morphological stage most people agree, except for Iwasawa, who  
20 somehow see it earlier. But almost everybody else agrees, in fact  
21 everybody else agrees, you can see the gonads at 56 and some of their

90

1 animals were at stage 67. I'm not sure what that is; I assume it's a  
2 juvenile.

3 So, that they didn't -- so, what they did was to treat throughout  
4 that period. So, for you to say that the critical window for sex  
5 reversal is -- in their study starts at 44 is inaccurate, because the  
6 treatment was continued through the critical window.

7 If I could just read into the record here what their results were  
8 calculated from this paper and I have the paper with me if anybody  
9 would like to see it, 1990, if they begin at stage 44 and treat all the  
10 way until they sacrifice the animals, they get 100 percent ovaries.  
11 That's true up until stage 50. Starting at stage 51, they start to get  
12 ovotestes.

13 There is actually a very nice picture in the literature of what an  
14 ovotestis is in review paper by Witschi, which I have with me, where  
15 you can see frank male gonad and frank, well-developed female  
16 ovaries. This is in an adult where it is easy.

17 So, you get ova testis beginning -- when you treat between  
18 stages 51 and 54 in this Villapando paper and then starting at stage  
19 55, begin treating out to somewhere between 56 and 67 you have  
20 either ovary or testes.

21 So, in this case, the critical window for sex determination

91

1 agrees completely with the previous studies by Chang & Witschi and  
2 so, you have to be careful whether the exposure is a continuous  
3 exposure given during critical period. So, I have to say I disagree  
4 with you that the results are inconsistent.

5 It is true that the Japanese group has sex differentiation of the  
6 gonads seen a little bit earlier, but they are the only paper.  
7 Everybody else can't see any sex differentiation until stage 56.

8 DR. BRECKENRIDGE: Thank you very much for that  
9 comment.

10 As I said, I'm not an expert in this area, but actually, the  
11 critical window portrayed on this chart is derived from the textbook  
12 chapter under by I Witschi, where he -- well, the blue line represents  
13 the data from Iwasawa, but the textbook chapter by Witschi has this  
14 little experiment where he is dosing in two successive day intervals.

15 He describes the feminization occurring in that period of time.  
16 He uses a different stage notation in that text chapter, so maybe you  
17 can help to clarify that for me?

18 DR. KELLEY: No. I think I'm just telling you that Villapando  
19 agrees completely with Witschi, that's all I'm saying and I that's -- I  
20 also think that the white paper graph of this is somewhat misleading  
21 and should be corrected.

1 DR. BRECKENRIDGE: Thank you.

2 DR. ROBERTS: Were there any other aspects of presentation  
3 or public comment on Dr. Ashby, Dr. Breckenridge or Syngenta  
4 otherwise?

5 All right I would like to thank you, gentleman, very much for  
6 your presentation today and the information that you have given to the  
7 panel and your discussion and dialogue with the panel. I would like  
8 to now go to a break for about 15 minutes and then we will continue  
9 with our public comment.

10 (Brief Break.)

11 DR. ROBERTS: Welcome back from the break. Our next  
12 public commenter is Dr. Tyrone Hayes, who is here on the behalf of  
13 the University of California Berkeley.

14 Welcome Dr. Hayes.

15 DR. HAYES: Welcome. My name is Tyrone. I first want to  
16 thank the panel for allowing me the opportunity to speak.

17 I'm from the University of California Berkeley and I am the  
18 Director of the Laboratory for Integrated Studies in Amphibian  
19 Biology. I'm currently Associate Professor, Professor as of this  
20 coming semester and I have been involved in studying amphibian  
21 developmental endocrinology for at least the entirety of my

1 professional career, even including my Bachelor's work, Bachelor's  
2 thesis work at Harvard where I conducted studies looking at the  
3 effects of temperature on growth development, metamorphosis and sex  
4 differentiation in wood frogs.

5 I then completed by PhD in the University of California  
6 Berkeley, under the direction of Paul Licht (ph), where I studied the  
7 role of steroids and thyroid hormones in growth development  
8 metamorphosis and sex differentiation. I then completed a post- doc  
9 of Child Institutes of Child Health and Human Development, again  
10 studying molecular and biochemical mechanisms of hormone action in  
11 amphibian development.

12 I want to start -- what I'm going to do today is first continue  
13 introduction with some of my affiliations and funding sources. I also  
14 might point out I'm not used to sitting down when I lecture. I'm  
15 usually a little more animated and so are my slides. So, it will be a  
16 very visual-based presentation. So, you might position yourself, if  
17 we could get the lights down.

18 After introducing all of my affiliations, I will give a brief  
19 introduction in my general interest in amphibian -- in my general  
20 interest in amphibians, in terms of their developmental  
21 endocrinology, the work that led up to my work in endocrine

1 disruption, if that's what we're going call it.

2 Then I will go directly into the studies that we've done with  
3 atrazine and describe in detail the methodologies that we have used to  
4 measure and document the endpoints.

5 Then from there I would like to put the work into broader  
6 context and talk to you about trying to establish cause and effect not  
7 just using my work but using work from the open literature and then I  
8 will close.

9 I understand that there might be pause for questions along the  
10 way and I'll certainly stop to address those, if that's okay with The  
11 Chair.

12 First I would like to introduce all of my funding sources. I  
13 have been funded by Novartis and Syngenta Ecorisks, however you  
14 want to call it. I've had funding from the National Science  
15 Foundation through multiple grants. My students have been funded,  
16 several of them through the Howard Hughes Medical Institute. I've  
17 had funding from the World Wildlife Fund, Jones Foundation,  
18 Homeland Foundation, the Rose Foundation, the Capour (ph)  
19 Foundation and The National Geographic Society.

20 I have listed at the bottom my own name, because I have also  
21 donated money from various awards and companies that I was

95

1 involved in to research and to personnel working in my laboratory.

2 So, those are my affiliations, not all of them necessarily contributed  
3 to the work that I'll present today.

4 This is also a list of the students have been involved in the  
5 work either directly or indirectly. The ones underlined were students  
6 who have coauthored work such as the National Academy paper that  
7 was discussed, the paper in Nature, the paper in Environmental Health  
8 Perspectives and I will discuss some of the other student's work that  
9 is also relevant.

10 I also want to point out that during the time I worked with  
11 Novartis and the Eco Panel, I was certified my laboratory good  
12 laboratory practices. Kathryn Benz was the person who did the  
13 training and so, my laboratory operated under the same -- I think it  
14 was called GOP operation, at least in terms of the QAQC and all that  
15 in terms of my certification.

16 That being said, one of the things that has fascinated me  
17 personally and professional about amphibians is the accessibility. I  
18 think growing the up as a child and continuing now, the ability to see  
19 what you are looking at newly fertilized egg that's about the size of a  
20 pin head.

21 In a few hours it turns into this, a few hours later depending on

1 species, you are now looking at an animal that is beginning  
2 neurulation, an animal that is about to break free of the egg jelly and  
3 begin swimming and depending on the species two months, eight  
4 weeks or sometimes several years later you get a mature frog.

5 What is fascinating about the animal is the ability to watch  
6 developmental events, including fertilization, first cleavage,  
7 gastrulation, neurulation and then the metamorphosis process.  
8 Primarily, the animal is accessible because there is no egg shell,  
9 because there is no membrane, there is no yolk sac.

10 It's not, you know, it has no placenta. It is not inside of the  
11 female when it is developing. That same accessibility makes it a good  
12 study tool also makes amphibians susceptible to chemicals such as  
13 endocrine disrupters or environmental pollutants that otherwise --  
14 other animals might have membranes or egg shells to protect them  
15 from being exposed to those chemicals.

16 In particular compounds that might mimic or interfere with  
17 hormones are significant, because many of the changes that we talked  
18 about including metamorphosis and sex differentiation and growth  
19 and development are regulated by some of the same hormones that  
20 people are concerned about.

21 In that regard because of not only sensitivity during

1 development but also because the animals are in land and they are  
2 also in water, they have a permeable skin, compounds might have or  
3 do have I believe, greater accessibility to the animals. So, you might  
4 see what might be low-dose effects that other animals might not  
5 experience.

6 The other interesting thing about amphibians, of course, is that  
7 they have very accessible end points that are regulated by the very  
8 hormones that we are interested in.

9 So, what are you looking at now is a larvae, this is a  
10 hyperlowase or leap frog. That's the adult and it is undergoing  
11 metamorphosis -- the climax of metamorphosis in and four days and  
12 that process is controlled by thyroid hormone.

13 Thyroid hormone is a modified amino acid and some of the first  
14 studies to establish thyroid hormone and thyroid gland, were available  
15 as early as 1912 by Gudernosh (ph), who, of course didn't have access  
16 to crystallized thyroid hormone, but fed thyroid glands from horses to  
17 tap poles. Really, as far as I know, first discovered the role of  
18 thyroid hormone in metamorphose.

19 Estrogen, depending on the species has a role. As you have  
20 heard, it can induce feminization, complete sex reversal. It can make  
21 a genetic male *xenopus laevis* grow ovaries and turn into a functional

1 female. That we have known at least since Gallian's studies 1953 and  
2 finally androgens in at least one tissue and -- we'll talk about others --  
3 is known to regulate the larynx or the voice box in *xenopus laevis*, as  
4 have you heard. We have known this at least since 1986 from Darsey  
5 -- Darsey Kelley's work.

6 Part of the reason I put this up is in addition to pointing out  
7 that these are -- as you will see, these are visual endpoints we can  
8 easily assess.

9 I also wanted to make the point while we're concerned about  
10 new problems, endocrine disruption, for example. I have heard the  
11 terms, "Emerging Science" and "New Endpoints" used. These are  
12 really endpoints that endocrinologists and developmental biologists  
13 have been well characterized and we have known for quite some time  
14 the role of hormones in many of these things and in fact, and down to  
15 the molecular level and the genes that are involved in many of these  
16 processes. So, they are not new points, new endpoints.

17 We may be applying that to studies in a different way, but it is a  
18 well-established science.

19 One of the animals I got interested in for a while is this one.  
20 It's *Hyperlissargous* (ph) and again, what you are looking at is a male  
21 and we know that because he is green. As you will see later, females

1 are different. You see here the vocal sack, which is androgen  
2 dependent feature like the larynx that you would only find in a normal  
3 male. The animals is unique in that the males and females are  
4 different colors.

5 This will show you in a minute this secondary sex character in  
6 the female is hormone regulated and it is regulated by estrogen.

7 For example, this is work I did with Karen Menendez (ph) that  
8 we published several years ago. You are looking at animals now,  
9 digital photograph 1, 2, 3, 4, 5, 6 days. That's the same individual;  
10 that's a control. If testosterone is applied, there is no effect, but if  
11 estrogen is applied, you can see the spotting. You can induce the  
12 female coloration prematurely or you can induce it inappropriately in  
13 males. One of the other things that we showed while studying the role  
14 of hormones in these animals, you are now looking at the underside --  
15 that's a control. It's a newly metamorphosed animal, that's an animal  
16 exposed to .1 nanograms per male testosterone and then 1 nanogram --  
17 and I'll show you another view.

18 You looking at premature induction of the vocal sac in a  
19 juvenile male. You can also induce this feature to develop in a  
20 female. So, again we have now we have androgen assay. The animal  
21 as estrogen assay on its back, an androgen assay on top on its bottom.

1 In all frogs you can use the back end if you will as your thyroid assay.

2

3 So, here is a control tadpole at two weeks of age, two months  
4 later the animal should look like that, but if you block thyroid  
5 hormone, for example, this two month old animal is the same as this  
6 animal, but at the same stage as this young tadpole.

7 So, what is interesting is -- and what we have been trying to  
8 develop in my laboratory over the last -- oh, I guess six, seven years  
9 now are easily measurable endpoints that are endocrine regulated by  
10 the three hormones that we're interested -- thyroid hormones,  
11 testosterone or androgens -- in this case testosterone and estradiol.  
12 This is just one assay. In fact, we patented this assay. This is just  
13 one assay that we have used, because we can treat the animals and we  
14 don't know have to know what we're looking for. We get all three  
15 hormones, both agonism and antagonism.

16 I'll give you an example of the utility of such as assay. This is  
17 work with Karen Menendez and Nigen Noriega, both former students  
18 in the lab. That's a control. This is animal treated with estradiol.  
19 The point I'm going to make I'm here -- I'm going to show you several  
20 compounds. The point I'm about to make is that when we have an  
21 assay that's estrogen specific in an amphibian but not only estrogen

101

1 specific, the specificity of the response, meaning the estrogen  
2 responds to and the types of estrogens that it doesn't directly correlate  
3 with estrogen's known to be functional in humans.

4 For example, estrogens that will induce mammary cancer cells  
5 to divide will also induce these color changes. I point that out,  
6 because I'm going to come back to this at the end and also -- someone,  
7 I think it was Dr. Vandercrack (ph) stated yesterday that if we know  
8 something about the human androgen receptor we can assume that the  
9 atrazine won't bind the frog androgen receptor and I'm kind of making  
10 opposite statement that knowing something about frogs will tell us  
11 something about animal's environmental health and public health in  
12 general.

13 In my mind, this meeting is about much more than amphibians  
14 but there are broader implications. So, moving along, here is --  
15 ethanol, estradiol, here is now a synthetic estrogen, that's used in  
16 birth control pills, so it's relative to humans. It gives a positive  
17 effect.

18 Here is diethyl, still the straw. It's a nonsteroidal synthetic,  
19 very potent estrogen, very potent in the frog. Here is OPDDT. Again,  
20 known to bind the estrogen receptor, not a steroid, not a hormone, but  
21 a pesticide that gives a positive effect.

1           And most interesting if you give estrogen in combination with  
2 tamoxifen, you can block the effect. So, here we have an antiestrogen  
3 that's important in humans in treatment of breast cancer, but it also  
4 shows a similar, positive response in a frog.

5           So, what I wanted to do is give you an idea of in general the  
6 kinds of work that we have been doing and how they might relate to  
7 the problem that we're faced with now.

8           So, let's get on with the atrazine thing. I probably don't have to  
9 tell you this. This is structure of atrazine. If you look at it there is  
10 no reason to think that it might interfere with any of the hormones I  
11 mentioned. It certainly doesn't look like a steroid.

12           It is an herbicide used with monocrops, corn and sorghum. It's  
13 been used for 40 years. We use something between -- the biggest  
14 numbers I have seen are 150 million pounds per year. The smallest I  
15 have seen are 60 million pounds per year in the U.S. It's used in more  
16 than 80 countries. As you know, it's a pretty major problem and one  
17 of the major, if not the most significant, most common contaminant in  
18 water as we'll get to.

19           What I'm going to tell you about now is, I'm going to go through  
20 the methodology that we use in my laboratory. I'm going to talk to you  
21 about the laboratory model we used initially to assess the effects of

1 atrazine. This work started when I was a part of the Eco Risk Panel,  
2 back in 1998 we started to work.

3 Then I'm going to talk to you about how we use those endpoints  
4 in *xenopus laevis* to develop comparative studies and ask do we see  
5 this effect across amphibians. How have we have modeled that? I  
6 want to point out too, with the comparative studies, because this has  
7 come up before, the goal wasn't in that initial study to do a full-dose  
8 range. The goal was to identify an endpoint to decide if we can go  
9 onto number three and do the field studies.

10 The goal was to take a native American frog, identify an  
11 endpoint and then move and to do studies in the wild, and we'll talk  
12 about that. The next thing we went on to do after doing our field  
13 studies and some work that we haven't published, yet we have done  
14 field simulations that I think address many if the problems that come  
15 up in the white paper and certainly some of the uncertainties  
16 associated with the field studies. Then, finally one ongoing study  
17 that I will tell you about that really brings the field back into the lab.  
18 So, something like, I guess, a reverse microcosm or something.

19 So, the laboratory model -- what I'm going to do now is I'm  
20 going to tell you a little bit about our procedure. Somebody said  
21 something earlier, I think Dr. Ashby, about things hadn't been

1 replicated. That's not true.

2 Typically, in my laboratory, we do a series of treatments. What  
3 you are looking at here, for example, represented by the rectangles  
4 are negative controls. We have an untreated control and an ethanol  
5 treated control. So, it is not true that all of our treatments contain  
6 ethanol.

7 We always have at least one ethanol treatment and we can talk  
8 about why that is, if you wish. Then we initially tested several doses  
9 of atrazine, .01 all the way up to 25 parts per billion or 25 micrograms  
10 per liter. Then we had a series of what we call "Positive Controls."  
11 We had a T-3 or thyroid hormone control to look at potential -- to  
12 have something to compare it or look at potential thyroid hormone  
13 like effects or antagonist effects. We had an estradiol positive  
14 control and a dyhydral testosterone positive control. All these  
15 treatments are replicated three times minimally, three times with one  
16 experiment.

17 We color code and do double blind analysis in everything in my  
18 laboratory. So for example, the stock solutions for these treatments  
19 might be made by Nigel Noriega and I might receive a series of  
20 numbered vials, 1 through 10 or whatever that adds up to -- 3, 8 --  
21 yes, 1 through 10, and then I will pull the numbers off those,

105

1 recording them and I will color code each one with some unique  
2 combination of colors.

3 So that Nigel does not know which solution is which and then  
4 two other personnel -- in this case Atiff Collins (ph) and Mendoza  
5 might do the dosing from the solutions to the animals. So, they have  
6 no idea -- in some cases the students have no idea what the treatments  
7 even are. They just know that they are distributing five colored vials  
8 of solution, making five carboys (ph) of solutions, dividing that up  
9 into five similarly colored tanks using five similarly colored nets,  
10 etcetera.

11 As each animal metamorphoses, the technician or student  
12 involved in harvesting those animals gives it a number. In this case,  
13 99 XLAZTR was the experiment we conducted with Novartix. So,  
14 each animal is assigned a unique number, so when analysis is done,  
15 all we have is number. So, if I go back and I analyze the larynx of the  
16 gonads, all I have is animal number 99 XLATZ546.

17 At most, I can trace it back to a color. I would have to go to --  
18 or back to a number and then I would have to go to yet another person  
19 to figure out which solution was solution Number 3.

20 So, that allows everybody to be able to do the analysis blindly.  
21 So, using that kind of setup -- and we'll talk later. I mean, we do

1 three-day static renew -- that actually started with Novartis Syngenta  
2 Ecorisk. We used to do renewal everyday. We do three-day static  
3 renewal now and we'll talk my feeding and things later.

4 The endpoints we examined in the initial study were mortality.  
5 We examined development, growth and metamorphosis. We  
6 determined the state and size of the animals on a regular basis. We  
7 documented the time of metamorphosis, both the time to form the  
8 emergence as well as to time the complete tail absorption and the size  
9 at metamorphosis.

10 And number two is thyroid hormone dependent. So, if a  
11 solution made the animals metamorphosis too slowly, then it is a  
12 antithyroid effect, too quickly, then it's a thyroid-like effect.

13 Number three, we analyze gonadal differentiation which in  
14 xenopus is influenced by estrogen not androgen. In some species  
15 androgens will make 100-percent males, not xenopus laevis. Estrogen  
16 will give you 100-percent females if administered properly and we  
17 analyze that endpoint. In number four, we looked at larval growth,  
18 which, as I said, androgen dependent -- or laryngeal growth, which is  
19 androgen dependent.

20 So, a compound and made the androgen grow big. It is acting  
21 like and androgen and compound that inhibited the larynx is somehow

1 interfering with androgen action.

2 Atrazine did not affect larval growth development or  
3 metamorphosis. Mortality as always in my laboratory, was about 90-  
4 percent. We don't accept anything below 85-percent and it is rare that  
5 we get 85-percent.

6 DR. KELLEY: You mean viability?

7 DR. HAYES: Viability. I'm sorry.

8 Mortality was average 10-percent and we don't accept anything  
9 greater than 15-percent. We found atrazine inhibited laryngeal  
10 growth in males.

11 What I'm going to do now before I show you all the data is I'm  
12 going to show you all the steps we went to to validate the  
13 methodology.

14 As I said, you are looking at now a picture of a stage 58 animal  
15 drawing. The gonads at that stage and a cross section through the  
16 larynx which I will talk to you. This is just to illustrate the laryngeal  
17 growth is androgen dependent. This is just a cartoon to show  
18 androgen -- demonstrating that androgen causes laryngeal growth.  
19 We did transverse serial cross sections -- not in tadpoles, but it was  
20 easier for me to draw a tadpole and what that means is we took  
21 sections in the direction and in the plain that you are shown here and

108

1 the muscle that we measured is the dilator larynges, which I will show  
2 you in a little more detail in a minute.

3 We measured the largest cross sectional area in the end and our  
4 final analysis, we only measured one side. We did not measure both  
5 sides and I'll show you why. You are looking at a dissected  
6 larynx. It has been stained; it is not normally that color. This muscle  
7 here, which I will blow up is the constrictor. This muscle is the  
8 dilator, so this pulls the glottis open; this constricts it. There it is  
9 blown up and what it is essentially is a hollow box with an opening or  
10 glottis that is controlled by these two muscles. This one is the dilated  
11 larynges again. So, now you are looking at a slice through it and you  
12 can see that it's this hollow box. There is the opening, the lungs  
13 would be back here.

14 This is now looking down on the larynges, so now are you  
15 looking at the dorsal or the top of the larynx. That's the bottom.  
16 That's is actually the parathyroid glands on the bottom of the larynx.  
17 And the sections that we examined were, as I said, transverse serial  
18 cross sections. I'll show you exactly which once they were, taken  
19 from the larynx about a third of the way through the dilated larynges.  
20 Now, looking at a series of sections and the section we analyze in  
21 each animal would have been the left side some where between this

1 one and this one. And in the end, we ended up doing it based on  
2 shape. I will show you all of the analysis we went through to decide  
3 that.

4 First question is can we really pick out the largest section and  
5 can we do it objectively?

6 Most of this work involved Ollie Stewart (ph), who was in the  
7 laboratory at the time. What you are looking at now is one exercise  
8 where I believe we took 20 micron serial sections throughout the  
9 larynx and then we had Ollie in the pink go through all the slides,  
10 through every single section and pick out the largest section.

11 So, that is his choice and then we had him go through -- he  
12 went through and measured every single section to figure out the  
13 absolute largest and then we analyzed the data -- a subset of the data  
14 asking, is his choice of the largest section statistically any different  
15 from the actual largest section. i.e., in going through and measuring  
16 every single section.

17 We found he was as good at picking the largest section, going  
18 through visually inspecting and picking the largest section as actually  
19 measuring every single section and picking it that way.

20 The next thing we did was we took a series of slides, I think  
21 several hundred slides. I'm showing you two examples and I used

1 tape to cover-up the slides. Ollie then picked the largest section. We  
2 measured it. Ollie picked the largest section and we measured it, then  
3 I taped all the slides, gave them back to him again, told him they were  
4 a different set of slides, had him pick the largest section again.

5 We did that for several specimens, which amounted to  
6 reviewing hundreds of slides. This just shows an example of how  
7 close he came to picking the same section each time and the total area  
8 after three times he could look at it and say, look you are giving me  
9 the same slides over and over again. So, we stopped the exercise.

10 I mean, you could telling that they were the same. The other  
11 thing we did is we taped over a series of slides that contained DHT  
12 estradiol controls, females and asked them to go through several  
13 hundred slides representing several specimens and treatments and  
14 pick out the largest. In other words, given a bunch of slides, we are  
15 asking him, can you pick out an androgen treated animal out of bunch  
16 of randomized slides? He was able to do that.

17 The next question we asked was is cross sectional area enough.  
18 I should point out this work was done while I was part of the Ecorisk  
19 panel. So, this was all available to the Ecorisk, overseen by the  
20 Novartis Syngenta Risk Panel.

21 For this exercise, we did frontal serial sections, because it

1 created fewer sections. Then, we measured every single section of the  
2 larynx and multiplied by the length to actually generate the full  
3 volume of the left and right of the larynx. In other words, maybe the  
4 cross section area wasn't enough to tell you enough about size.  
5 Maybe we needed to estimate volume.

6 It turns out you can get data this way but it was quite time  
7 consuming. With the sample size that we're trying to do, it was quite  
8 impractical.

9 The next question we asked was, is the largest section  
10 representative? We did a series of things. We looked at land marks  
11 such as, for example, always measuring a section that looks like this,  
12 as opposed to always measuring a section of dilated that looks like  
13 this, we measured the exact middle section. We measured the total  
14 length as a measure.

15 This shows the largest section, geometric center and the two  
16 landmarks. This horizontal line represents the length of the larynx.  
17 This represents the cross section area of the left and the right and  
18 essentially we showed that we got the same data, the best data by  
19 picking out the largest section, that no landmark was better able to  
20 give you same data. In fact, we ended up using a landmark because,  
21 because the largest cross section is always -- at least at

1 metamorphosis in the same section of larynx.

2           So, in summary, the analysis of laryngeal volume was  
3 impractical, at least in the way that we were doing it, by counting  
4 every single section and calculating it that way. The choice of largest  
5 cross sectional area, we deemed accurate, relative to looking at  
6 landmarks, repeatability, ability to do it blindly, ability to pick out  
7 the androgen positive controls. The choice of largest section was  
8 repeatable. In other words, we could blind and give back the same  
9 samples and get the same data.

10           The analysis of landmarks provided the same information; there  
11 was no landmark that gave us a better measure than picking the  
12 largest section.

13           We looked at the left side versus the right side, which again I  
14 don't have to go into detail. What I'm showing here are the untreated  
15 -- the negative controls, the positive controls, the atrazine treated  
16 ones. The blue are the females, the yellow are the males. We show  
17 there was no difference between left and right side. I'm going go  
18 through the data in detail.

19           We corrected the data for snout-vent length, which I don't think  
20 is appropriate and certainly, collecting for body weight is not  
21 appropriate, because of the alatri (ph) and because the larynx is

1 growing completely androgen dependent and not necessarily relative  
2 to body size. But we were asked to do it and we did it. It gives you  
3 the same data, same effects.

4 This shows the final data set which I'm going to go through in  
5 more detail. This shows that same data set corrected for snout-vent  
6 length.

7 What I'm going to do now is I'm going to talk about starting  
8 with one. I mean, I'm going talk about both of the data sets from the  
9 PNAS. One of the things that will become clear at the end of my talk  
10 now it that we're now talking about a repeat or replication of the first  
11 experiment.

12 The first experiment was down under the auspices of the  
13 Narvata Syngenta Ecorisk Panel. That was done in -- began in 1988  
14 and was conducted throughout -- into 1999.

15 The studies that we published in the National Academy of  
16 Sciences are now a second and a third study. So, that is my -- now a  
17 third replication of a study that had three replicates of each treatment  
18 within. Does that make sense? If anything I say doesn't make sense  
19 throw something at me.

20 So, what you're looking at now is laryngeal size, the largest  
21 cross section of the area of the left. For example, I noticed in --

1 whose was it -- Dr. Ashby's talk, he had really big larynges and then  
2 he had mine down there, but that's actually only half the larynx,  
3 because we only looked at one side and that's stated in the paper.

4 Here is the average size for males. Here is the average size for  
5 females. This is in our controls, in our ethanol treated controls.

6 What I'm going to show you now -- individual data points and I  
7 believe, if I'm not mistaken, you are looking at 10 males per replicate  
8 -- the points that you are looking at. I want you to notice a couple of  
9 things. One is, this is the average for control males, these are the  
10 individual data points and they are evenly distributed. I'll show you  
11 this in a different way. Half are above average and half are below  
12 average. If we look at our positive control, the dihydrotestosterone,  
13 on average they are larger. There is the mean, but more of the  
14 animals are above where a controlled male would be, if that makes  
15 sense.

16 I'll show in it a different way in a minute. Now, are you  
17 looking at the atrazine in parts per billion, .01, .1, 1, 10 and 25.

18 What I want you to notice in the red bars, on average they are  
19 smaller, starting at one part per billion. What I also want you to  
20 notice is that more of the animals are falling below the mean. In other  
21 words, the distribution is changing. If you do a test of homogeneity

115

1 variance you will find that you fail, which means that an analysis of  
2 variance is not appropriate of the data.

3 DR. KELLEY: Which replicate are these data from?

4 DR. HAYES: This is from work from -- this is work from the  
5 PNS paper.

6 DR. KELLEY: Okay. So, this is two and three?

7 DR. HAYES: Two and three? Yes.

8 DR. KELLEY: You just described it?

9 DR. HAYES: Yes.

10 There is similar data at well presented in the report that I sent  
11 to you, Syngenta, from the original study.

12 What are you looking at now -- the same data, just presented in  
13 a different way. The blue show the proportion of animals above the  
14 mean, above the mean for the control males in untreated controls and  
15 in ethanol treated controls.

16 They are normally distributed -- about half are above average  
17 and half are below average. If you look at the DHT treated animals --  
18 so the estradiol treated animals, there is no males, they are all  
19 females. You are only looking at male data now, that's why there is a  
20 blank.

21 For the DHT treated animals, 90-percent of the animals are

1 above average. In other words, 90 percent of the animals are above  
2 where a control male would be, above that average.

3 If you look at the atrazine treated animals, increasingly more  
4 animals are below average with the dose of atrazine, such that at 1  
5 part per billion, 80-percent of the animals are below average, the  
6 average for controls and 90-percent of the animals at 10 and 25 are  
7 below average.

8 The reason we looked at the data this way were a couple  
9 reasons. One was, I wanted to know if it was inappropriate. We  
10 conducted non-parametric analyses and the question we were asking,  
11 which I think is more relevant to the population and I welcome the  
12 panel to comment. The question we are asking is: If you are in an  
13 atrazine contaminated environment, what proportion of the animals  
14 would be effected?

15 It is like the example I like to use with my students is -- it's  
16 like the GREs and SATs. Nobody know their raw score, you know,  
17 your percentile. You want to know how am I doing relative to  
18 everybody else. How am I doing if I weren't exposed as a population.

19 Maybe I'll stop now if there are questions. Does that make  
20 sense how we're doing the analysis?

21 DR. KELLEY: Could you go back to the previous slide?

117

1 So these ends are actually different in your --

2 DR. HAYES: No, they are not.

3 DR. KELLEY: They look different.

4 DR. HAYES: Well, there are points on top of points. There is -

5 -

6 DR. KELLEY: So, the end is this -- could you tell us the ends?

7 DR. HAYES: It is ten animals per replicate, if I recall  
8 correctly.

9 DR. KELLEY: But there is definitely more than 10 in the  
10 control group. I just counted up the dots.

11 DR. HAYES: One, two, three -- my recollection is, it was ten.

12 DR. KELLEY: There's definitely more than ten.

13 DR. HAYES: It was more than ten. I apologize.

14 DR. KELLEY: But the ends -- you believe the end were equal  
15 for these groups?

16 DR. HAYES: Yes.

17 DR. KELLEY: Actually, there a lot more than ten. Okay,  
18 thanks.

19 DR. HAYES: I can go back and give you that number exactly.

20 The question was could we repeat these data especially given  
21 that the doses were so low and, in fact -- this is now the same data

1 that I just showed you, so here are the males and here are the females.  
2 There is the one part per billion. In addition to repeating, what we  
3 decided what we wanted to do as well, is to look between these two  
4 doses to try and determine if there was a dose response.

5 So, we looked at a zero dose which was our control. Then we  
6 looked at .1, .4, .8 and one part per billion and then we also looked at  
7 25 and 200 in this second experiment. And again, we got the same  
8 kind of effect where it appeared starting at one part per billion, we  
9 got the reduction in laryngeal size.

10 What you are looking at now is another representation now of  
11 both experiments, the percent above the mean -- I believe this figure  
12 was also published in the PNAS paper. This is percent above the  
13 mean relative to atrazine dose and starting at 1 part per billion, there  
14 seems to be a threshold effect where 80-percent of the animals are  
15 below average, starting at 1 part per billion.

16 If we put that on a log scale, it looks like there is a linear dose  
17 response. These are the same data just on a log scale and these are the  
18 two experiments.

19 We looked at -- we tried to do or did correlation analysis to  
20 look for dose response and we did not get a significant P value,  
21 greater than .05. That is what you are looking at on the left. We also

1 did a Kendall's coefficient of rank looking at whether or not there was  
2 a dose response in the proportion of animals effected. In other words  
3 we asked, with increasing dose is there a greater number of animals, a  
4 greater proportion of the animals affected by atrazine. We got a  
5 significant P value, less than .01.

6 So, in our final analysis, there were no differentiates between  
7 the left and the right side of the larynx and so we chose to use only  
8 one side as a timesaving device.

9 DHT treated males and females had larger larynges and Inova  
10 could not identify an effect of atrazine and, in fact, because of the  
11 heterogeneity of variance, Inova was not the appropriate test to use  
12 and that's why they moved to the non-parametric analyses. The non-  
13 parametric tests revealed an effect of atrazine above -- I should say 1  
14 part per billion -- we're starting at one part per billion, greater  
15 proportion of the animals were below average.

16 And further analysis revealed a dose effect with increasing  
17 atrazine dose increased the number of below average males.

18 What I would like to do now is talk about the second ends point  
19 and go through some of the terminology. In fact, I have both  
20 terminologies here on the slide.

21 atrazine produced intersexes or hermaphrodites in 16 to 20

1 percent of the exposed animals.

2 I will show you now the types of gonadal abnormalities that  
3 have been discussed here in detail and give you the terminology that  
4 we have settled on. It is true that we changed terminology between  
5 the xenopus and the rana and that's because the effects manifest  
6 themselves in a different way between the two species, as I'll show.

7 First, what I want to do is tell you how we determined the sex  
8 of the animals. Again, I want to go through some of the procedures  
9 we use in the laboratory. You are looking at now a male and female.  
10 I will show you how we can tell. This is a freshly dissected kidney of  
11 a male and female -- that's fat body in the male, the gonads actually  
12 there and in the female the gonad is actually there. They are  
13 transparent. Unless you fix them and this is why we fix in Buren (ph)  
14 solution. It contains Petrac acid and it turns them yellow.

15 I can show you this is if same animal if you look at that set of  
16 pigment. That corresponds to a set of pigments over here. This is the  
17 exact same animal. The transparent gonad is here and there is the  
18 testes. The testes is short in a male. It is only about a third the  
19 length of the kidney. It's typically smooth or unlobed and xenopus  
20 lacks pigment.

21 In the female, again, the is the exact same animal now stained

1 or preserved in Buren, the ovary is usually this long -- or is this long  
2 structure, extends the entire length of the kidney. It is ventral-medial  
3 to the kidney. It is lobed and it has black pigment or melanin  
4 interspersed throughout.

5 The way we identify the gonadal abnormalities in mammals  
6 is the following. We were trying to do a study where we took animals  
7 at different stages, determined their sex and froze them immediately.  
8 We were going to save them for hormone analysis. In other words, we  
9 had a set of animals that we could not preserve in Buren. If you go  
10 back, these almost invisible things, Ollie, Stewart and Erin Vonc (ph)  
11 and I -- these almost -- I was trying to sex animals based on these  
12 almost visible structures.

13 So, I question whether or not -- how accurate I was. We took a  
14 subset of animals where we did just this. I went through and sexed  
15 them, we preserved them in Buren, and then I went back and sexed  
16 them again to determine if I were correct. We did about a 100  
17 animals, if I recall and blindly. I didn't know which ones were which.

18 I was right 100 percent of the time with controls and somewhere  
19 between 15 to 20 percent of the time, if it was an atrazine treated  
20 animal, I was incorrect. I would call it a male and later finds out it  
21 was -- I'll show you. That was initially how we discovered the

1 problem.

2           Here is what we do in my laboratory for any experiment, for  
3 every experiment. Once the animals are harvested, the students or  
4 personnel who have been involved with the project have only access  
5 to a color. The ones who have been doing the animal care have no  
6 idea which treatments were which. They might know, for example, in  
7 this case I'm going give you example of four animals that came out a  
8 blue tanks, four animals that came out of a red tank. When the  
9 animals are preserved that's all that is known. They are given an  
10 individual number and they will know the tank number that they came  
11 from.

12           Those animals as they come out would be given a specimen  
13 number, something that has the experiment name on it, followed by  
14 the number for that specimen and then one person involved in the  
15 project such as Nigel Noriega would go through and look at each  
16 animal. He would look at an animal like this and say that one is a  
17 male. He would record they are male. What I'm going to do is, I'm  
18 going to show you how we double-check on each other throughout the  
19 process. I'm also going to show you how we define the abnormalities  
20 that have been discussed here today.

21           He might go through a second animal. Again that's a female.

1 So, you see the long structure the entire length of the kidney, the  
2 black melanin interspersed throughout the lobes. He would call that  
3 one a female. That one would be a female, you've got another male.  
4 Then he would get to something like this. It is long almost entire  
5 length of the kidney, but it is got no pigment.

6 I don't know if you can see it from there but it is lobed. It  
7 doesn't meet all the criteria that we use when we're storing somebody  
8 as a female. So, he would record UO, or what we call unpigmented  
9 ovary in this case. He might get another animal that looks like this.

10 I'll go through it later, but it has got what look like multiple  
11 gonads. We initially call that lobed testes. I think this is the same  
12 thing that Dr. Carr calls discontinuous gonad. And you might get  
13 something like this that has -- what looks like unpigmented ovaries as  
14 well as lumps of testes and he would record that as a hermaphrodite,  
15 sometimes they are recorded as intersex or if it's confusing, we might  
16 record it as a question mark and go back and review it.

17 A second person, in this case, Roger Leu (ph) would come by  
18 and review all of those animals blindly, again, and usually one of the  
19 people involved is not involved in the project at all. It is somebody  
20 working on something else and then we might have a trainee,  
21 somebody who is now sexing animals for the first time, again, do it

1 blindly.

2 Then I, personally, sex every animal that is used in my  
3 laboratory. That's in excess of 10,000 animals per year. Then we'll  
4 put the whole thing together. We'll go through and we'll ask, for  
5 example, are there animals that we all disagree on and then we'll  
6 review all of those animals together especially, for example, this is  
7 Mable Choy (ph) if it is a novice and somebody new, that's our  
8 process of teaching them how to identify gonads.

9 Any questionable gonads, any ones that get a UO or an LT or an  
10 H, and a subset of normal males and females, all under go histological  
11 analysis for confirmation.

12 A subset of males and females would be analyzed. In the case  
13 of xenopus -- in rana, we do everybody. A subset of males and  
14 females, all anomalies are analyzed histologically. The histology  
15 looks like this.

16 In a normal male -- this line represents transfer cross section.  
17 It's like slicing a salami. The difference in color is now because it is  
18 a stained that we use when we do the microscopy. The testes is  
19 always solid at stage 66, at metamorphosis. You can see the blue  
20 rings of connective tissue, so the testicular lobules are starting to  
21 differentiate. The ovary typically has this ring of connective tissue,

1 ovarian vesicle and it is hard for me to see from all the way back here,  
2 but those are melanin granules, the same melanin granules that you  
3 see there in the ovary.

4 An animal like this -- in this case I'm going to do a section this  
5 way and I'll blow up now these sections. In this case, if you are close  
6 enough, you can see these are all individual gonads that have been  
7 sectioned serially, that don't seem to have any connection.

8 So, there are discontinuous gonads or as Dr. Carr calls them,  
9 discontinuous gonad or as we have called them, lobed gonads or  
10 broken testes is another term that students have used, but it is the  
11 same thing. There is no female, there's no ovarian tissue apparently,  
12 either morphologically or histologically. It looks like multiple testes  
13 resident in a single animal.

14 Sometimes you get animals look like this. In this case, there is  
15 a testes on one side, maybe a little ovarian tissue and an ovarian  
16 ovary on the other side. There is a cross section to confirm that.  
17 That's the renal artery. There is the testes, there is an ovary with the  
18 ovarian vesicle. Sometimes we get rostral coddle arrangements but  
19 not necessarily in any order.

20 So, sometimes we get, in this case testes on top with ovaries  
21 coddling and what I'm going to do now is I'm going do one side

1 sagittal. So, you can see there is kidney and there is the ovary. You  
2 should see -- you could see a section there. This it would be the next  
3 section.

4 Again, that's the section through your ovary and so, we're  
5 basically slicing up. When you reach the interior portion, you start to  
6 get into testicular portion that's actually quite well differentiated.  
7 You can see these holes are actually testicular lobules starting to  
8 form. On the other side, if we do a transfer section, that's a large  
9 testes that might be an ovarian vesicle starting in that testis. It is not  
10 clear and further down it is ovarian.

11 Here is an animal that has truly a mixed hermaphrodite, as we  
12 call it. So, it has two testes followed by two ovaries, a large testes  
13 and more ovary. We did serial cross sections; I'm only showing you  
14 representative ones. There is the fat body which is always attached to  
15 the interior portion, two testes followed by two ovaries, there's the  
16 large testes, there's ovary and the two ovaries at the back again --  
17 testes ovaries.

18 So, what we would do now with this data set is fill in the  
19 histology, confirm that the histology matches the gonadal mythology.  
20 Then we go through and we cross everything out that appears in  
21 controls.

1           By cross out I mean we have defined abnormalities as  
2 morphologies that show up in treatment groups once the data decoded  
3 that never appear in controls. We defined it that way. So,  
4 discontinuous testes, unpigmented ovaries -- I will tell you in a  
5 minute, we have found a very low frequency in some controls, but  
6 discontinuous of the lobe testes and the hermaphrodites, we have  
7 never in over 10,000 animals per year at least for the five years.

8           And in every study, this is how we identify the abnormalities.  
9 Does that make sense? Questions?

10           So, in xenopus, anyway, there is this range of abnormalities.  
11 For example, what we call the single sex polyglobulism in a multiple  
12 testes or lobe testes. The lateral hermaphroditism, where sometimes  
13 we have one gonad one side and one on the other, an ovary on one  
14 side, testes on the other, as shown here.

15           This is an animal with anterior or posterior hermaphroditism or  
16 caudal/rostral. We also get these mixed hermaphrodites. But again,  
17 these are all morphologies that we've never seen in controls, using the  
18 methodology I just described to you.

19           This was another one of the morphologies, the unpigmented  
20 ovary. So, the structure looks ovarian but it lacks pigment. We have  
21 according to the PNAS paper. We've now identified in a study, 3

1 animals out of 300 -- 3 controls out of 300 that have the unpigmented  
2 ovary. As you will see, it's a very low frequency in controls, but  
3 there is that difference now.

4       What I reported in the PNAS paper, the 16 to 20 percent  
5 hermaphrodites, were those morphologies that I just showed you and  
6 I'm going to show you now, a larger data set. I'm going to show you  
7 the individual types of abnormalities that we found per dose. What  
8 appeared at the time -- and my thinking now is a little bit different,  
9 but here is a male and here is a female. It seemed that these  
10 abnormalities were kind of in a continuum. So, for example, a normal  
11 male has one pair testes, then we have these animals that have  
12 multiple testes, but they are clearly male they are not hermaphrodite.  
13 They have multiple testes, but all of the testes morphologically and  
14 historically -- all of the gonads appear to be testes.

15       So, the next intermediate step is an animal that has both testes  
16 and ovaries. Not necessarily in this arrangement. I mean, there can  
17 be caudal rostral or lateral or mixed. But this animal clearly has some  
18 male characteristics as well as some female characteristics. In closer  
19 to the female is this sort of unpigmented ovary. It is a structure that  
20 doesn't look testicular.

21       On histological cross section it looks ovarian, but it's lacking

1 pigment and in some case it is very shallow -- has very shallow lobes.

2 In other words, it is not as lobed as a normal ovary would  
3 appear and lacks a pigment obviously.

4 What I'm showing you now are a series of colored boxes. I'm  
5 going to show you graphs. So, basically, I'm giving you the legend  
6 before I show you the graph. You are going to be looking at males in  
7 blue, females in yellow.

8 There are going to be stacked bar graphs, then you are looking  
9 at -- from here it looks purple, but that is a hatched bar that is mostly  
10 blue with yellow lines. This is most male-like of the abnormalities.  
11 In the middle it is a hatched bar that is equally yellow and blue and in  
12 the end, it's a fine hatch that's yellow with thin blue lines.

13 So, in other words, I tried to make this continuum as it  
14 appeared to be in the morphologies.

15 This is now a data set looking at multiple doses focusing on the  
16 low doses and one high dose. Again, males are in blue and the  
17 females in yellow. One thing to notice is that in most of the groups,  
18 with maybe one or two exceptions, it appeared that the females are  
19 about 50 percent and the males are under 50 percent.

20 So, in other words, it looks like the abnormal gonads might be  
21 due to inappropriate development in males as opposed to a mix of

130

1 males and females. Part of the reason that -- the hypothesis that these  
2 are males that are being effected and not females is in part because at  
3 least with steroid hormones, females seem to be pretty determined.

4 In other words there is not a steroid mixture that will make in  
5 xenopus females turn into males, but estrogen will make males turn  
6 into females. So, the male sex differentiation seem to be more plastic  
7 and the fact that you had -- we had close to 50 percent females in the  
8 treatments and some positive males also suggested that it was the  
9 males that were being reversed, if you will, and not the females.

10 What I'm going to show you now, because you can't really see  
11 the numbers when they are stacked up there with the real sex animals  
12 is I'm going to show you the lobe testes and hermaphrodites and the  
13 unpigmented ovaries without the proportion of males and females.  
14 Those are the doses.

15 These are the proportions of the different types of  
16 abnormalities over the different doses and as I have said we have now  
17 identified about one percent in one experiment of the animals had the  
18 unpigmented ovaries in a control.

19 Questions?

20 DR. KELLEY: Would you like to comment on the dose  
21 response aspect of this slide?

1 DR. HAYES: I will. I have a whole section I have prepared,  
2 where I will address to the host response on both to the larynx and the  
3 gonads. So, if we can hold to that?

4 DR. ROBERTS: There are a couple more questions. Dr. Green  
5 and then Dr. Denver.

6 DR. GREEN: Could you clarify what the significance is of an  
7 unpigmented ovary at this stage?

8 Do you know that it will not go on to become a normal  
9 functioning ovary?

10 DR. HAYES: I have absolutely no idea. All I know -- well, I  
11 will show you some -- a little more data later. All I know is that in  
12 this case it very rarely shows up in controls and it seems to be  
13 associated with -- in this case, atrazine treatment. I will show you  
14 another intriguing experiment that suggests a possible mechanism.

15 But I don't know the significance of it. I don't know what it  
16 turns into. I'm going address that in just a minute.

17 DR. ROBERTS: Dr. Denver.

18 DR. DENVER: Do we know that it is not atrophied intrarenal  
19 tissue?

20 DR. HAYES: Historically unpigmented ovary, historically, it's  
21 an ovary, it looks like an ovary. It lacks pigment, it's very shallow.

132

1 Their vesicle is small, and occasionally, the medulla is still in tact.  
2 So, it looks somewhat undifferentiated, but it not adrenal tissue or  
3 intrarenal tissue.

4 DR. ROBERTS: Dr. LeBlanc.

5 DR. LEBLANC: Dr. Hayes, I think it is in your PNAS paper  
6 that you referred to a 16 to 20 percent incidence of abnormalities?

7 DR. HAYES: Yes.

8 DR. LEBLANC: This is not what we're looking at here?

9 DR. HAYES: This is a larger data set now. This is three times  
10 the number of animals.

11 DR. LEBLANC: Is it included in here?

12 DR. HAYES: It is included in here, yes.

13 DR. LEBLANC: Thank you.

14 DR. ROBERTS: Any other questions?

15 Go ahead, Dr. Hayes.

16 DR. HAYES: So, now I'm going to address my ideas and some  
17 data concerning the mechanism a little more thoroughly, but I want to  
18 introduce it here for a couple reasons.

19 The hypothesis that we been working on primarily because of  
20 the data showing increased estrogen in rodents exposed to atrazine,  
21 the data from Sanderson et al., showing the up-regulation of

1 aromatase and some other data I'll go into, we explored the possible  
2 induction of aromatase as an explanation for the two effects that we  
3 observed.

4 As you know, normally in the testes testosterone is synthesized  
5 and secreted. We have proposed that atrazine in the testes and  
6 perhaps now in the brain -- I have to admit we haven't looked in the  
7 brain, that atrazine -- based on these previous studies, which I will  
8 address later -- atrazine induces aromatase and then two things occur  
9 -- we proposed. One is testosterone levels are lowered and as a result  
10 you are demasculinized.

11 This, for example, might explain the decrease in laryngeal size.  
12 If these animals had low testosterone as a result of aromatase  
13 induction and in turn, the estradiol production might account for the  
14 feminization of the male gonads. I'll give you some evidence for that  
15 as well for the role of estrogen.

16 I do want to disagree with one thing that came out in the white  
17 paper. This doesn't necessarily mean you would find estrogen  
18 circulating in the blood of an individual.

19 I think Dr. Ashby gave the excellent example of the male brain  
20 in mammals being masculinized by estrogen, but it's because  
21 aromatase is expressed in the brain and the estrogen is made locally.

1 That estrogen doesn't necessarily circulate. I think there are other  
2 examples where you will find that as well. So, you might expect it,  
3 but it is not necessary, I don't think.

4 I don't think you have to find circulating estrogen necessarily  
5 to support the hypothesis that aromatase was induced. So, there is our  
6 proposed mechanism. So, we think it would work something like this.  
7 You are now looking at *xenopus laevis*, 48 to 56. These are figures  
8 from Newquip and Phoper (ph).

9 What I'm going to show you now is what happens in males on  
10 the top what happens to females on the bottom. The gonads  
11 differentiate in our laboratory -- gonads are differentiated historically  
12 somewhere around -- as early as stage 52 and certainly by 54 you can  
13 start to find differentiated gonads. I'll talk a little more about  
14 critical periods later. The larynx at some point -- and we haven't done  
15 studies in the larvae, but the larynx at some point in females  
16 presumably doesn't grow because there is not androgen. In males,  
17 androgen from the testes presumably caused the larynx to grow,  
18 because males and females are differentiated, at least in terms of the  
19 size -- not the fiber number but at least in terms of the cross sectional  
20 area at stage 66.

21 That's what males do. What we're proposing in atrazine-treated

1 males is that the testes starts secreting androgens, but it gets  
2 converted to estrogen -- again, not necessarily leaving the gonad and  
3 results in the production of ovaries in the animals. And as a result of  
4 this impairment of gonadal development, androgen is not available  
5 and the larynx has impaired growth.

6 In part, we believe that that is why you don't get fully  
7 feminized animals, because there has to be some testicular tissue  
8 differentiated to give you the testosterone substrate.

9 We believe that's why you get the mixed gonads, because some  
10 of the gonad has to have differentiated to make the testosterone that  
11 gets converted to estrogen that then feminizes, perhaps, the slower  
12 developing parts of the gonad.

13 As evidence for this -- I'll give you a lot more at the end -- this  
14 is the data that we published in the PNAS paper. Plasma testosterone  
15 levels -- that's in a controlled male, that's compared to an atrazine-  
16 treated male and these are adults by the way. These are adult animals  
17 that were exposed to atrazine. The atrazine-treated males is  
18 significantly -- atrazine-treated males are significantly -- have  
19 significantly reduced testosterone levels but not relative to  
20 controlled males and aren't different from controlled females.

21 This is a One-Time static measure of testosterone that was done

1 on an animal that was -- or animals that were euthanized.

2 The other evidence, to be quite honest, isn't strong. I'll address  
3 it further. We didn't publish this data for that reason. This is a  
4 measure of aromatase activity, using a treated water assay. You are  
5 looking at control males and that's background -- a blank tube will do  
6 that. Then you are looking at some measurable aromatase activity but  
7 at -- incredibly variable in terms of the individual animal's response.  
8 I will show you the individual data points and tell what you we have  
9 been trying to do about that later.

10 The next question that we went on to ask, this started the about  
11 three years ago -- is a question that has been asked here multiple  
12 times, is what happens to these animals?

13 So, in fact, when I was still part of the panel, we were doing  
14 some other experiments, treating some animals with atrazine and we -  
15 - I guess what we're calling a grow-out. We grew some of these  
16 animals out. The design was the following.

17 White arrows indicate controls, animals that weren't treated.  
18 So, again, same design. We had three tanks of each of these, three  
19 tanks of animals that were treated throughout larva development. So,  
20 a total of six tanks. Are you going to see two separate experimental  
21 regimens. Here is another set of tanks that were treated with

1 atrazine. In one case, the animals were treated the only into  
2 metamorphosis and then grown out to see if we can reverse the effects  
3 and then we had controls set up to compare to. We also had some  
4 animals that were treated for 18 months, not as larvae, but treated  
5 after metamorphosis and then we had animals that were treated both  
6 as larvae and are still being treated in my laboratory today. They are  
7 about three years old now, I guess. At 18 months we evaluated all  
8 these groups.

9 So, we took a subset of animals. Some of them were sacrificed  
10 at metamorphosis -- a third of the animals. A third of the animals are  
11 grown up for 18 months and a third of the animals remain in my  
12 possession in this design.

13 If we look at -- now at 18 months animals that were not treated  
14 as larvae and then grown up after metamorphosis for a year and a half,  
15 the larynx is quite different. You can see the dilated larynges here.  
16 Female controls -- the example I'm showing you right now is actually  
17 in terms of snout-vent length exactly the same size as male. This is  
18 male and female exactly the same snout-vent length.

19 If would look at animals that were exposed for -- throughout the  
20 larval period for two months and then not exposed for a year and a  
21 half, they typically have -- well smaller larynx. Although we couldn't

1 find a statistical difference between the two, they typically have what  
2 looks like an impaired laryngeal development. If we look at animals  
3 that were not exposed as larvae and in exposed as adults, they  
4 typically look like this. Then if we look at animals that are exposed  
5 throughout they typically have very small larynges that, in fact, aren't  
6 different from females statistically, but also aren't different from  
7 males.

8 We looked at other things however. Somebody asked about this  
9 yesterday. We looked at the nuptial glands. So, males by -- in our  
10 laboratory anyway -- by as early as three to four months post  
11 metamorphosis, typically start to develop their breeding glands. If we  
12 do histology through those glands, it is looks something like this. I  
13 believe cretinized structure as compared to similar section of female.

14 And we also looked at the coctolabiles (ph). Here is a control  
15 male. That's what a female would look like at 18 months and 30  
16 percent of the atrazine-treated males at 18 months effectively, look  
17 like a female.

18 So, we would have animals, for example, with breeding glands  
19 sometimes, that also had a female-type concha.

20 And there is a big problem. We were talking about fertility and  
21 all those things. I will tell you what the problem is in a minute.

1           So, normally what should happen in a male is that testosterone  
2           from the testes, by 18 months, should cause the larynx to grow and the  
3           nuptial glands to develop and the cowayka shouldn't grow -- that's  
4           estrogen dependent and testosterone should promote spermatogenesis  
5           in the testes. For example, you can see nest of germ cells developing  
6           at various stages in the lobule of this animal that I have shown as  
7           representative.

8           With atrazine, you get a whole host of effects. One is often the  
9           testes of animals that were treated with atrazine that we can identify  
10          as males, look like this. I have to say I can't interpret it for you. It  
11          looks like lobules that are filled with debris or junk.

12          They have varying degrees of sex reversal or oocytes in the  
13          testes in cases, although we have not seen it as high as the levels that  
14          we saw some of the other labs talking about. We have seen a very low  
15          percentage.

16          Some of the males -- again, some of the animals that have  
17          nuptial glands also have protruding cowayka which suggests that  
18          there is circulating estrogen. I should say though -- I'll show this  
19          later, we have never been able to measure circulating estrogen in a  
20          male, atrazine treated or control. We have never been able to detect  
21          it.

1           They have impaired larynges as well, 30-percent of the animals  
2           that we looked at. The problem with the fertility is that we started off  
3           with three replicates of each of these treatments I showed you, 30  
4           animals per replicate. Approximately a third, as I said were  
5           sacrificed at metamorphosis, a third were sacrificed at 18 months. So,  
6           we have 30 animals left and in the treatment groups, the most we have  
7           are two males. There are no sex chromes. I can't prove to you the  
8           animals completely transformed into females, but we'll start doing  
9           trials injecting with ACG, giving females to the animals that appear to  
10          be males and eventually all of the animals will start to lay eggs. Of  
11          the two males we have -- well we can't do anything with two males.  
12          They have never fertilized an egg. It has been difficult trying to get  
13          fertility data on animals that have grown out.

14          What I'm going to do now -- I don't know if we want to stop for  
15          questions -- now, I'm going to take what we have learned in *xenopus*  
16          *laevis* with we have a few other things going on, but primarily, I'm  
17          going to take what we have learned from *xenopus laevis* now and we'll  
18          talk a little bit about the leopard frogs the *rana pipiens*, the laboratory  
19          work first.

20                 Questions?

21                 DR. ROBERTS: Dr. LeBlanc.

1 DR. LEBLANC: The incidence of cowayco for that you  
2 reported -- I think you said is was 30 percent in atrazine treatment?

3 DR. HAYES: Yes.

4 DR. LEBLANC: Is there any incident in the controls?

5 DR. HAYES: Not that I recall. I mean, those animals are also  
6 still available. They are preserved whole and they can easily be  
7 reanalyzed.

8 DR. LEBLANC: In this set of experiments, it is a single  
9 concentration of atrazine you worked at.

10 DR. HAYES: Yes. I believe it was 25 micrograms per liter that  
11 we used.

12 DR. LEBLANC: It is higher level?

13 DR. HAYES: I would have to double check. I can't --

14 DR. LEBLANC: But it is a higher level in the range that we  
15 discussed?

16 DR. HAYES: Yes.

17 DR. ROBERTS: A couple more questions. Dr. Kelley and then  
18 Dr. Denver.

19 And just -- Dr. Hayes, for planning purposes and the audience,  
20 my intention is to go until about 12:30 and then take a break for an  
21 hour for lunch and then resume with Dr. Hayes's presentation.

1 DR. HAYES: What time is it now?

2 DR. ROBERTS: That would be another 20 minutes.

3 DR. HAYES: Is there going to be a break point around in  
4 there?

5 DR. ROBERTS: Yes.

6 Dr. Kelley and then Dr. Denver.

7 DR. KELLEY: The studies with the adults where you treated  
8 them for 46 days with atrazine, what time of year was that?

9 DR. HAYES: Do you remember? Spring? Melissa says spring  
10 and she was in charge.

11 DR. KELLEY: I bring this up because, as you know, the levels  
12 that you get are lower than levels we would get from summer animals.  
13 Our experience has been that they maintain an endogenous -- at least  
14 the population we have maintains an endogenous circumanal rhythm  
15 in the laboratory. So, I think -- but these animals were all done at the  
16 same time of year?

17 DR. HAYES: They were done at the same time and I have a  
18 whole section where we are going to show seasonal cycles. We have  
19 animals we've carried out for a year. We also have shown that the  
20 animals ordered from Nasco have much higher testosterone levels  
21 than our Berkeley animals and we've also shown -- those are measured

143

1 during the day, Melissa, those ones in the PNAS paper -- that's  
2 daytime?

3 We've also shown that if we measure them at night, levels can  
4 about four or five times higher. So, now we do all of our  
5 measurements at nighttime. So, that was a daytime measure in the  
6 spring from Berkeley animals. You will see different levels when we  
7 go further.

8 DR. KELLEY: Could you tell us, in the PNAS paper, what the  
9 time was to metamorphosis? How long did it take your animals to go  
10 through from treatment to metamorphosis?

11 DR. HAYES: It is about 45 days. I have that number in another  
12 part of the talk I think the average is 45 days from that paper, but I  
13 have a number that I'll show you for sure.

14 DR. KELLEY: One last comment is that the dilated larynges  
15 does not control the glottis with the arytenoid disk.

16 DR. HAYES: Oh, I'm sorry.

17 DR. KELLEY: In rana it does, but in xenopus it is made with  
18 the arytenoid disk.

19 DR. HAYES: Thank you.

20 DR. ROBERTS: Dr. Denver.

21 DR. DENVER: I'm just trying to get a handle on the

1 characterization of the replication. You mentioned that the  
2 treatments were replicated three times with 30 animals per replicate.  
3 That was an N of 3 or --

4 DR. HAYES: That's three tanks with 30 animals in each.  
5 That's been done one time in the Syngenta study. There are two  
6 studies published in the PNAS. There is animals that were sampled  
7 from --

8 DR. DENVER: Right. I'm not asking about the number of  
9 times you did the study. Within a study, do you consider that an N of  
10 3, because you remove animals and characterize the gonadal  
11 morphology on 10 at a time, I think you stated?

12 DR. HAYES: In -- no. In the animals that we move out in  
13 terms of gonadal morphology, everyone one of them sexed by gross  
14 morphology. Every single animal is examined. Histology is done on  
15 a subset of control males and females and on any animals that get a  
16 question mark, a UO or any kind of -- what we call sex comments.  
17 Those animals all get confirmed by histology.

18 DR. DENVER: What I'm wondering is, do you consider that an  
19 N of 30? It is a pseudo replication --

20 DR. HAYES: When we do -- sorry.

21 DR. DENVER: Do you consider it an N of 30 if you take 10

145

1 animals from each tank?

2 DR. HAYES: No, because if you are doing ratios, each tank  
3 only has one number. So, each animal has no value. You will get 10  
4 percent of hermaphrodites, 40-percent this, bla, bla. Each tank only  
5 has one number, so each tank is a replicate as to sample -- it's an N of  
6 3.

7 For larynges it is different because you actually have a  
8 quantitative measure for each individual. We do statistics was a rank  
9 where we look at treatment by tank by individual -- or by sex as well.

10 DR. ROBERTS: I think Dr. Kelley has one more before you  
11 continue.

12 DR. KELLEY: Do you inter tank -- well, first of all I need a  
13 number. I need to know the -- or perhaps you will tell us this later,  
14 the number of your frank hermaphrodites in your treatment groups. In  
15 the PNAS paper, you lumped together the various gonadal groups that  
16 you saw. But the number where you saw both a clear ovary and a  
17 clear testes.

18 DR. HAYES: Those are the ones I showed there.

19 DR. KELLEY: Right. Now, did you have enter tank variability  
20 in that percentage of those frank -- what I call frank hermaphrodites?

21 DR> HAYES: Well, it is not exactly to the same per tank. I

1 don't remember off the top of my head how many are which, but those  
2 data can be made available.

3 DR. KELLEY: Did you have any tanks in which you had none  
4 and tanks in which you had a lot.

5 DR. HAYES: No. I mean, other than controls, no.

6 By the way, in the analysis that we did do, we do it by  
7 abnormalities and it is only been now that people have asked I have  
8 started to pull out the types of abnormalities. At the time of the  
9 PNAS paper, I believe the only things that we talked about separate  
10 were the discontinuous testes and what were clear hermaphrodites.

11 We've now been trying to differentiate and I've also been  
12 working with Al Beasley (ph) to try and differentiate the types of  
13 abnormalities and types of hermaphrodites.

14 So, with that as a starting point, we wanted to conduct  
15 comparative studies. In part, because maybe this was just a weird  
16 effect that we were finding in *xenopus laevis* and wouldn't occur  
17 across species. What you are looking at now is a phylogeny of anuran  
18 families. We decided to look at -- we won't talk about the high lid,  
19 but we decided to look at two species completely unrelated to *xenopus*  
20 *laevis* in part because if we found effects here in this major group  
21 here and here, then I feel like we can start to make some statements

1 about how generalizable it was to amphibians -- the effect, that is.

2 In part, we chose a high lid because Reeder, et al., had shown  
3 some effects in the field associates with atrazine with trepidations, I  
4 believe, and we chose rana pipiens, because it's also an animal that's  
5 accessible that we can breed in the laboratory, that we can also  
6 examine in the field and unlike xenopus, it responds to both  
7 testosterone and estrogen. It also has a dual response to estrogen,  
8 where very low doses do nothing, intermediate doses make 100-  
9 percent females and high doses of estrogen make 100-percent male.  
10 We thought it would be an interesting animal to look at for that reason  
11 as well.

12 The gonads are a little bit different compared to xenopus laevis.  
13 This is also at metamorphosis, complete tail resorption. I will also  
14 point out later that the gonads are different in terms of the level of  
15 differentiation depending on the population. What you are looking at  
16 now are -- is a male and a female. These are animals originally from  
17 Wisconsin. These are the animals that were reported in the Nature  
18 Paper and in Environmental Health Perspective Paper. I'm going  
19 show you cross sections to show you the differences. In the male, you  
20 can see at the testicular labials, the spaces are the labials maturing.  
21 Later I'll show you germ cells in some of those animals. In the female

1 you not only have the vesicle, but you can see oocytes, already in the  
2 cortex of the animals at metamorphosis. You don't do see this -- at  
3 least we have never seen this in xenopus laevis at metamorphosis, but  
4 you can already see the developing oocytes there.

5 I'm going to show you series of animals. Some of these -- I  
6 think these are all the figures that appeared in the Environment Health  
7 Perspective Paper. Most of the animals were not identifiable as  
8 problematic just upon gross morphology.

9 So, most of the animals, if they were identifiable looked like  
10 they just had a broken testes or lobe testes like we saw in xenopus  
11 laevis.

12 If you did a histology however, I'm going show you three points  
13 on this animal, three sections. It is clearly testicular, anteriorly. It is  
14 connected, so unlike the xenopus lobe testes or discontinuous testes,  
15 there is a connection here at this juncture.

16 In this particular animal, as you get towards the back, there is a  
17 large oocyte. We talked about testicular oocytes in this animals, in  
18 part -- I'm not sure if we should call them hermaphrodites, because  
19 they don't seem to have ovarian tissue. They seem to have testes with  
20 the wrong germ cell. You ought to have testes with oocytes. They  
21 don't seem to have testicular tissue.

1           Here is another animal that again looked like it just had  
2 multiple testes, but when you do the sections, it is clearly male.  
3 Those are developing spermatids inside that lobule.

4           A few sections more. It's clearly male, still. When you get  
5 towards the back, there is large oocytes and again, more and more  
6 increase in oocytes. This was always the arrangement. It was -- the  
7 animals were always male anteriorly and then more and more female  
8 as you progress back.

9           They always started out male. In this case, even spermatids and  
10 then became more feminine. Here is an animal -- and you know, a  
11 comment was made about 1 or 2 oocytes, here is animal where this  
12 section is testicular. I'll blow it up for you. This section is clearly  
13 testicular. The back end is completely filled with oocytes.

14           Here is another animal that has testes anteriorly and the testes  
15 posteriorly or caudally, are completely filled with what appear to be  
16 fibrogenic oocytes. So, I'll draw cross sections for you here and here.  
17 You can see now the sections between what appear, as I said, even to  
18 be fibrogenic oocytes, but would imply that there is circulating  
19 estrogen. I will address the dose response later. Right now I just  
20 want to present the data. We characterized a couple abnormalities.  
21 One is what we call "Gonadal dysgenesis," This was poorly developed

1 or poorly organized testes with closed lobules. We did find it at a  
2 very low percentage in controls, much higher in the atrazine treated  
3 animals.

4 You are looking at in the black, gonadal dysgenesis in the red  
5 testicular oogenesis. Again, I'm reluctant to call it hermaphroditism,  
6 because they appear to be males with oocytes, not animals with a mix  
7 of testes and ovaries.

8 One of the interesting things is it appears rana pipiens is  
9 supposedly XY, XX or male hetero and like in mammals, whereas the  
10 whole -- I'm sorry not like in mammals -- whereas the whole gonad  
11 appears to develop and become the ovary. It is stretched the entire  
12 length of the kidney. In males, it appears that the gonad develops  
13 anteriorly and some signal -- maybe testosterone, causes the posterior  
14 portion not to develop. It is not clear what that substance might be.

15 The implication with these atrazine-treated males is that this  
16 signal is not released or is blocked. If that signal is testosterone, the  
17 animal becomes demasculinized and as a result feminized. In other  
18 words, the posterior -- I'll go back -- the posterior of that gonad,  
19 which technically should now be signal-to-regress from the  
20 developing testes doesn't get that signal and by default appears to  
21 develop as female or at least to allow germ cells to develop into the

151

1 default oocyte, as opposed to inducing sperm.

2       Maybe now would be a good -- now, what I was going to do is  
3 go into the field studies. I just want to make the point that that  
4 original laboratory study, with the two doses was only designed to  
5 determine if there was an effect. If there was an end point the main  
6 goal was to identify an end point that we could assay in the field. So,  
7 when we talk about dose effects -- certainly in the paper, not now,  
8 don't claim to have done that and shown an inverted U, but certainly  
9 with the data points we have might suggest that.

10       DR. ROBERTS: Let me ask the panel then, if they have any  
11 questions regarding what you have presented so far?

12       If not, maybe this would be a good time to break before you get  
13 into the field studies. Let's go ahead and take a break for lunch for  
14 approximately an hour. I have 12:20, now let's reconvene at 1:30 and  
15 continue with your presentation, Dr. Hayes.

16       DR. HAYES: Thank you.

17       DR. ROBERTS: Before we continue with Dr. Hayes public  
18 comments, I just want to make a couple of housekeeping  
19 announcements.

20       One is, someone left some glasses in here yesterday, so, if you  
21 are missing some glasses, Shirley is hold them up. If you recognize

152

1       them, everybody look quick, check and make sure you still have your  
2       glasses. If you don't, they may be up here.

3               We have had a couple of questions about the camera here and  
4       what is the camera doing and that sort of thing. Just as a general  
5       statement, this is a public open meeting and people are permitted to  
6       take photographs as long as it does not interfere with the Panels's  
7       activities.

8               This particular case, this is an independent film company that is  
9       making documentary on atrazine issue.

10              This is not part of EPA taping the meeting or anything like that.

11              Let's now continue with Dr. Hayes's presentation. Are you  
12       ready to go?

13              DR. HAYES: Yes.

14              DR. ROBERTS: All right, great.       DR. HAYES: Before  
15       we start, just one point of clarification. I'm not -- as a teacher, I'm  
16       not normally nervous in front of an audience, but I'm talking about the  
17       larynx in front of the world's only expert. I was correct, it's 10 per  
18       replicate. There are three replicates, as Bob Denver and I were  
19       discussing, so, there are 30 points per line.

20              I just got shaken up there. So, as a point of clarification.

21              We were about to go into the field. We conducted controlled

1 laboratory studies, identified gonadal abnormalities and xenopus  
2 laevis and used that as it a design to describe gonadal effects of  
3 atrazine at two doses, .1 and 25 parts per billion in rana pipiens and  
4 now we are off into the field to determine whether or not we can  
5 identify gonadal abnormalities in field-collected animals and also to  
6 collect water samples and to identify whether or not any gonadal  
7 abnormalities detected are associated with atrazine contamination or  
8 other pesticides.

9       The first thing we did before taking off was asked whether or  
10 not the effective doses were ecologically relevant. In both rana  
11 pipiens and xenopus laevis, we see abnormalities at .1 part per billion  
12 or .1 micro gram per liter.

13       What I'm showing now -- atrazine levels. These are data from  
14 the literature in parts per billion. I don't know how to translate this,  
15 but the recommended application rate is 2.9 to 29 million parts per  
16 billion. That's 290 million times the level that we're using in the  
17 laboratory our studies. This is a range of levels gleaned from the  
18 literature. The first you are looking at are min and maximum levels  
19 reported in the literature in runoff. Temporary pools, permanent  
20 water and finally levels detected in precipitation, including snow and  
21 rainfall and this was just through an open literature search that I and

1 personnel in my laboratory conducted.

2 This is the level we're concerned about when looking for  
3 gonadal abnormalities or hermaphrodites, .1 part per billion. The red  
4 shadow indicates that all of the habitats would be at risk, based on the  
5 .1 part per billion level that we're examining, based on our laboratory  
6 studies in two species.

7 This shows the 200 parts per billion. I believe it's the MCL, if  
8 that's what it is called and this is the three parts per billion that was  
9 at least the drinking water standard at the time -- recommended  
10 drinking water standard at the time.

11 So, at the level we're concerned about, .1 part per billion, is  
12 considerably lower, 30 times lower than 3 parts per billion.

13 What I'm showing you now is an animation that I have made of  
14 a figure from the USGS. I believe it was produced by William  
15 Battaglin, who I am now collaborating with. Approximately 60  
16 million pounds into the Midwest. So, you are looking at the Midwest  
17 and US of the Missouri river. What he is showing here, he has  
18 conducted a two-year study, measuring atrazine levels in surface  
19 water at the sites indicated. This shows the 3 parts per billion, which  
20 is the EPA current standard and what you are looking at now is the .1  
21 part per billion, the level we're concerned about.

1           So, the idea here was to look at a map of measured atrazine  
2 levels and ask throughout the year -- and this is a study done over two  
3 years, throughout the year are there places where you would find  
4 water that exceeded the .1 part per billion that we were interested in?

5           And, in fact, you do almost completely throughout the year, but  
6 you would notice in each year there are spikes of atrazine in -- when  
7 you plot amphibian breeding seasons over these same areas you see  
8 there is a direct overlap, such that for example, at this site amphibians  
9 would be breeding at the rise of the peak in atrazine so that the larvae  
10 would be maximally exposed and metamorphose would occur right  
11 about the end of that spike.

12           The reason for this is atrazine levels, I guess, increase during  
13 first rains and that's when you see that spike. Of course, amphibians  
14 are also in these regions, typically breeding at first range. So, the  
15 timing -- the levels are there and the levels are there at a time when  
16 the animals would potentially be exposed. This was all just  
17 preliminary work that we did. Before, we didn't embarked on taking  
18 such a huge endeavor.

19           What we attempted to do was a large, natural experiment. You  
20 are looking at a map of atrazine use in kilograms per kilometers  
21 square, based on sales. These aren't actual atrazine measures, but we

1 used this map developed by William Battaglin to develop hypothesis.  
2 For example we can go to develop our hypothesis. So, for example,  
3 we can go to areas in Utah and examine amphibians as well as the  
4 water that they are in, expecting that would be in controls -- a control  
5 site, low atrazine and no gonadal abnormalities. We can go to  
6 counties such as here, where there is some atrazine use as a potential  
7 exposed site. In Nebraska, for example, we can go to sites with high  
8 atrazine use, expecting to find high atrazine contamination as well as  
9 hermaphrodites. We can go to sites such as Cherry County, where  
10 there is very little atrazine use and we would expect to find low  
11 incidence of hermaphroditism.

12 You are looking at the range of rana pipiens now, the true rana  
13 pipiens, although we'll discuss this in a minute.

14 The leopard frog, the northern leopard frog. We took off trying  
15 to stick to one parallel and -- well, and also following Iata (ph) you  
16 might recognize. Again, the idea was, each point we collected water  
17 and -- I'm sorry, Dr. Ashby errored. We collected 100 frogs from  
18 each site. There were sites -- if there were sites where we could not  
19 collect 100 frogs or frogs seemed to be rare or sparse, then we did not  
20 collect them, in fact, that's why we did not continue with the high like  
21 work with the tree frog work, because were only two sites where we

1 could collect 100 metamorphosis, not adults -- 100 metamorphosis,  
2 newly metamorphosis animals.

3       These are leopard frogs in the field. The animals did not die.  
4 Again, we did not collect 20, as Dr. Ashby said, we collected 100.  
5 There were no deaths they were euthanized immediately. Each  
6 collection took several hours. They were euthanized in benzocaine  
7 and preserved in Buren solution and then analyzed back in the  
8 laboratory.

9       There is myself, Mable, Kelley Hasten and Adrian Brown --  
10 were three of the students who accompanied. To give you some idea  
11 of where we looked -- here is one of the sites in Iowa. It is runoff  
12 from a cornfield, so you are looking at a runoff ditch there and there  
13 is the corn. We tried to look at a variety of sites. This is a nearby  
14 area that is protected. There is not a corn there, it a wildlife refuge  
15 that we have permission to collect on. Here is another site along a  
16 river that is not adjacent to corn fields.

17       Here are some of the atrazine levels. We had one site where the  
18 atrazine levels weren't available. The reason is the following. We  
19 had the analysis done by three laboratories. The analysis was always  
20 done blind. So, in other words, they got numbered samples, they had  
21 no idea where the water came from, the water was frozen immediately

1 and the samples were analyzed for atrazine by three laboratories.  
2 PTRL West, it's a private laboratory; it is the same laboratory that  
3 Novartis, Syngenta, Ecorisk was using when I was on the panel. They  
4 were also blindly analyzed by at the Iowa Hygienic Laboratory. So, a  
5 university laboratory and they were also analyzed by USGS  
6 Laboratory. So, a private, government and a university laboratory.  
7 We only accepted the data if the numbers came back within 10 percent  
8 of each other and at least one of the labs came back -- I'm sorry, two  
9 of the labs came back with nondetectable levels and 1 lab gave us a  
10 number of .2, I believe, for this site. So, we didn't use the data,  
11 because they didn't all match.

12 Here is one of the sites in Cherry County, Nebraska. There is  
13 no corn use, it is sand prairie. Here is one of the sites in Nebraska  
14 that is a corn field and .6 parts per billion atrazine was measured  
15 consistently at both sites. So, even though there is no corn use in  
16 Cherry County, Nebraska, there is atrazine contamination.

17 This is one of the sites in Utah. This is a site in Wyoming, in  
18 the North Plat River and this is a site, also in Utah, on a golf course in  
19 a county where there is local atrazine use. This was the only site that  
20 we analyzed that also had frogs that had nondetectable atrazine  
21 levels. atrazine was also detectable in Wyoming, which I'm going to

1 discuss in the North Plat, because it is not in the vicinity of corn  
2 growing areas. There has been some discussion about the site.

3 Back in the laboratory, we analyzed, we dissected and analyzed  
4 the animals. This is a typical male, that's a typical female. I'm not  
5 prepared to say that there are no effects in females. I'm saying with  
6 the methodology that we have been using for rana and xenopus, we  
7 don't detect abnormalities or and kinds of effects in females in any of  
8 the assays that we have used.

9 So we -- at this point, we have actually -- are only analyzing  
10 the males. At least in the assays we are using, we do not detect  
11 gonadal abnormalities or hormone abnormalities in females. This is  
12 an animal collected from the field. I believe one of the pictures that  
13 was in both the Nature and the EHP Paper -- I will below this up  
14 again. Very clear, testicular lobules with oocytes. In fact, if you do  
15 serial sections, it is not just a few, the entire gonad -- throughout the  
16 entire gonad, every lobule have one oocyte. I won't bore you with  
17 more pictures, I will just show you where we found hermaphrodites at  
18 some percentage. I'll show you the percentages as well.

19 One of the big surprises was the North Plat River in Wyoming.  
20 The site in Utah -- again, there is local atrazine use on the golf course  
21 here. These sites are all associated with an area of high atrazine use.

160

1 So, that's almost understandable. The one big concern was this one  
2 site in Wyoming where there is no atrazine use. To me it appeared to  
3 be quite a pristine place, it was actually a lovely place to camp, but  
4 also had the highest percentage -- highest proportion of  
5 hermaphrodites out of all the sites.

6 Here is what -- just an example of one of the animals. If you do  
7 the sectioning through not only testes with oocytes, but some of the  
8 testes actually have what appear to be ovarian vesicles resident in  
9 them as well.

10 So, what is going on? Let's sort of below that up a little bit.  
11 The North Plat River flows this way. Maybe everybody knew this. I  
12 didn't know it. The North Plat River flows this way, so that it is not  
13 atrazine traveling from Nebraska, but it originates in Colorado and  
14 I'm working now with William Battaglin. We're sampling in these  
15 areas in the spring, the USGSs and in the summer a joint effort  
16 between myself, my laboratory and the USGS. We will be exploring  
17 this source of the atrazine contamination there.

18 Can we blame atrazine? I mean, at this point, we're going to  
19 talk about the doses but all I can tell you is that at every site where  
20 you find atrazine above .1 part per billion, we find hermaphrodites in  
21 some proportion.

1           Here are those data, so we look at the hermaphroditism or the  
2           testicular oogenesis. They are probably not appropriately called  
3           hermaphrodites, because they don't have ovarian tissue. Animals with  
4           testicular oogenesis, we also had one site with animals that had  
5           predominantly testicular or gonadal dysgenesis. I will talk to you a  
6           lot about that site. This is actually -- we found out quite a bit about  
7           these animals recently. These are the atrazine levels associated with  
8           those sites. So, there is not -- I think like Dr. Ashby said, it almost  
9           looks like there is inverse correlation. We'll talk about those  
10          difficulties in a minute.

11          One thing that is interesting -- so now what you are looking at  
12          are the range of rana pipiens again, overlaid on the map of atrazine  
13          use. There is the route we took with all the sites. I can't see it from  
14          back here. I hope you can. There are a couple other things to  
15          consider. What I'm putting up now are ranges for other leopard frogs.  
16          It used to be sometime ago that rana pipiens was this huge -- the  
17          species with this huge range all across the United States. It was  
18          determined there were actually multiple species of leopard frogs,  
19          southern leopard frogs, northern leopard frogs, etcetera.

20          What is interesting is that now, instead of following just  
21          following Iadi (ph) we have formulated some other hypothesize and

1 we have funding now to follow drainages and river systems. What  
2 you are looking at now are some predictions of where we would find  
3 high atrazine contamination and again expect to find hermaphroditism  
4 associated with some of these sites and are you also looking at rivers  
5 now that we predict -- based on where they, are based on atrazine  
6 sales, we predict to have low atrazine contamination and we would  
7 predict low incidences of hermaphroditism. In other words, the route  
8 we took even, with the hypothesis that we had based on use, it was  
9 difficult to find an atrazine free site.

10 I don't think that's a weakness in the study. I think that says  
11 something about how widespread the problem could be. If you don't  
12 just find atrazine on the Cornfields -- I will show you in a minute -- it  
13 moves around quite a bit -- we have now permission to get into some  
14 of these head waters and some isolated lakes, if you helicopter -- from  
15 a guy who owns quite a bit of the water up there. So, that's one of the  
16 things we are doing now.

17 What I want to point out now -- it's quite interesting. These  
18 aren't my data, although I have manipulated the figure a little bit. A  
19 few months ago I met a woman named Rita Kenadia (Ph) who was  
20 finishing her Master's thesis and she -- working at SS State. She came  
21 to see me because what she was doing was -- let me go back. What

1 she was doing was she was working along these contact zones, using  
2 mitochondrial and nuclear DNA analysis. She was trying to determine  
3 if these were real boundaries. So, for example, this yellow should be  
4 rana blaireye (ph) and all this pink should be rana pipiens.

5 It turns out that coincidentally, this site that had the high  
6 gonadal dysgenesis, the animals that were unlike all the other rana  
7 pipiens -- one of the things she came to tell me was they are not rana  
8 pipiens, they are rana blaireye.

9 So, rana blaireye, which should be down here is now appearing  
10 at our site. I can't even tell if I'm pointing at the right thing from  
11 here -- appearing at our site in there in Nebraska.

12 So, there has been a range extension. What is more disturbing  
13 is these black circles up here, this should be the range of rana pipiens.  
14 This should be rana blaireye. What those gray circles indicate are  
15 animals that have random blaireye, mitochondrial DNA as well as  
16 rana blaireye, nuclear markers that she was looking at.

17 Now, we have done some work now, on this gonadal dysgenesis  
18 thing, this poorly developed gonad and we're actually formulating  
19 hypothesis now that it's actually a mechanism of resistance. We don't  
20 get hermaphroditism when we expose certain frogs. For example,  
21 certain sites in Utah, certain sites in Nebraska -- we don't get any

1 hermaphroditism at all. This is work subsequent to our EHP paper. I  
2 will show you what the animals look like. It appears that the animals  
3 which have a slow gonadal development, sort of metamorphosing with  
4 poor gonadal development, undifferentiated gonads, are resistant in  
5 part because they are metamorphosing after they leave the water -- I'm  
6 sorry, gonads are developing after metamorphosis or after they leave  
7 the water.

8 In one such population is the one down here from Nebraska,  
9 which shows the high gonadal dysgenesis. Let me just stop for a  
10 minute. Is that making sense at all or am I rambling?

11 So, we have animals like rana blaireye, their gonadal  
12 development seems to be delayed -- not seems to be. 100 percent of  
13 the animals seem to be delayed, either undifferentiated or small  
14 gonads, the kind of thing we called gonadal dysgenesis. They don't  
15 show hermaphroditism in response to atrazine. The hypothesis we're  
16 working on now is that they are resistant, because the gonads are  
17 differentiating. Essentially, the critical period has been shifted until  
18 after metamorphosis.

19 Now, the disturbing part about this figure is, everything that  
20 she has measured in here, in Nebraska, all the way into South Dakota,  
21 the mitochondrial DNA is rana pipiens. So, these are all rana pipiens.

1       Pipiens. The nuclear markers that she is looking at -- and these sites  
2       with high pesticide use, are all random Blaireye.

3               So, in other words, rana pipiens is gone. The females are  
4       clearly all rana pipiens because the mitochondrial DNA is rana  
5       pipiens, but they appear to be choosing or maybe only having a choice  
6       of these random Blaireye males, which we have identified as a  
7       potentially resistant species to atrazine. So, it is just one of the  
8       things that we are working on now. She is now joining my lab for a  
9       PhD. to combine development in endocrinology, et cetera, with the  
10      population and genetics and kinds of things that she is doing. But it  
11      speaks to the impact. There was something about robust populations  
12      came up. One problem is, if you go to a site -- and we went to some  
13      sites where you find no frogs. It is hard to say, well, whether or not  
14      the frogs were affected by pesticides, there is no way to tell why they  
15      went away once they are gone.

16              At the same time, just because you find frogs there and I think  
17      we had discussion about robust populations, it doesn't mean that they  
18      are what they used to be. She is now looking, for example, at the  
19      possibility of genetic bottle necking. In this case it could also be  
20      hybrids, which may or may not be driven by pesticides. There has to  
21      be ways to get at those kinds of answers.

1           What we're going to do now is -- we have had these control  
2 studies where we can identify end points. We can try to identify  
3 mechanisms and probe into those mechanisms. Although control these  
4 aren't real studies. We have now gone and tried to do -- I mean, they  
5 aren't real world, I should say. We have now gone into the real world  
6 and tried to do a study where we looked at whether or not the effect  
7 occurred and whether we -- and whether or not there was an  
8 association of contamination. But there are a lot of things that are  
9 uncontrolled in the field.

10           So, now what we're going to do is look at some of those  
11 uncertainties in the field. I'm going tell you how we try to make more  
12 real laboratory experiments to try to look at what some of these  
13 factors may be and to try to control some of these real factors that we  
14 might be interested in.

15           One problem is -- you are looking at a field. I'm going to show  
16 you this again. In the winter, just as an ice starts to melt, when the  
17 rana pipiens breed -- this is a field in Nebraska. Everything is  
18 covered in water. Even though you might try to look at plots, one  
19 where there is atrazine in use and one where there is not, the water is  
20 almost continuous at least part of the year. So, this is point .3 parts  
21 per billion. You are actually looking across an organic farm that's

1 across the street from the corn farm that we work in. This is in the  
2 winter time, so this is when the levels should be their lowest. If you  
3 look at rain fall in the area, it is .4 parts per billion. Again, we're  
4 concerned about .1 part per billion. That's where we're seeing effects  
5 in rana pipiens as well as in xenopus laevis.

6 The water in this field pond, just behind the site is at .9 parts  
7 per billion. That's the same water that's taken up and irrigated -- even  
8 though atrazine is only applied twice's year at the site. the field pond  
9 is drainage from the corn field, which is then reapplied to the field so,  
10 essentially some level of atrazine is applied throughout the year.

11 Again, this is run off that eventually ends up in the field pond  
12 and applied back. The other problem is even in one ditch, this is the  
13 same ditch from one day to the next you can go from 15.3 parts per  
14 billion to .6 parts per billion. This came up with some of the  
15 Syngenta Ecorisk studies where one time measurements. Even  
16 multiple measurements don't give you the full range of what animals  
17 are exposed to.

18 Again, those are some of the difficulties in trying to make those  
19 dose response curves we have been discussing especially with field  
20 data. I think we may have to settle -- unless we can think of ways  
21 around it, for good laboratory studies that show that the animals don't

1 develop this way normally in the lab unless they are exposed to one of  
2 the pesticides you are interested in and then just looking for the  
3 association or presence or absence.

4 I don't know any other way around that. Again, here is another  
5 example. This is the ditch I was just showing you that's running off  
6 of the farm that we're working on, it's running into a wildlife refuge.  
7 This is a protected area. The other end of it runs through a pipe and  
8 floods the organic farm.

9 If we wanted to set off and do plots based on use, it is almost  
10 not possible, so there are a lot of uncertainties.

11 There are an other problems. For example, in Nebraska, I  
12 guess, there is a law that requires you to post pesticides that you are  
13 applying to your field. So, here's a sign -- the farmer -- all the  
14 farmers have given us permission and allowed us to work on the land.  
15 I have blanked out number of his site.

16 What he posted here -- I guess, what is posted here inside this  
17 tube if you look in there it says, this report lists the pesticides applied  
18 to this field. You can actually go and look at everything that has been  
19 applied to the field.

20 The problem is -- so we did. We looked. We looked in both  
21 years that we went out. The problem is, sometimes -- for example,

169

1 here you have the compound thyfluomide (ph). It's got these -- what  
2 do they call it -- these EPA numbers and then the next year those same  
3 EPA numbers show up on this government document. It shows up  
4 with tedinfurous (ph) and syflufeuren (ph).

5 In fact, the farmer said he never applied thyflumoide, so he  
6 doesn't know why it's listed there on his -- on his record.

7 Here is atrazine. atrazine was applied twice. Each time it is  
8 applied it has a different EPA number. I don't know if that means a  
9 different formulation or not and the farmer wasn't able to tell us that.  
10 The other part of the problem is even if you can figure out what is on  
11 this cornfield, it is often times adjacent to another field. So, there  
12 might be 10 things on the Cornfield and 10 things in the cornfield and  
13 there might be another ten things on the soy field and the frogs are  
14 being collected from here.

15 So, I guess, all I'm agreeing with is the point in the White Paper  
16 that if you find abnormalities in the field, how can you know that  
17 those about abnormalities were caused by atrazine when there are so  
18 many other -- in this case, at one site pesticide is used.

19 I would argue some of the strength is we can raise those  
20 animals in the laboratory from that very population. We can raise  
21 rana -- we have rana pipiens year round. We can raise them as

1 controls and know that they don't develop that way in the laboratory  
2 unless they are exposed to atrazine. Still when you get to the field,  
3 again, how can you know?

4 So, what we did was -- this is a list of all the herbicides used at  
5 the site. This is a list of the fungicides used at the site. This is a list  
6 of the insecticides used at the site. We had the USGS, as well as Iowa  
7 Hygienics Laboratory, PTR West was too expensive.

8 We had them analyze several sites, the sites that we were  
9 interested in, Utah, Wyoming and two sites in Nebraska. We had them  
10 analyzed for all of these chemicals. They had methods. I can get you  
11 those. I'm not a chemist, but I could get you the methods they used.  
12 We analyzed the samples for all of these come pounds and the idea  
13 was and the idea still -- we're still working on this, is that we test  
14 each one of these compounds.

15 If it's really atrazine that's causing the gonadal problems,  
16 atrazine will show up, the other compounds won't. Then we did  
17 something else too. We not only tested them singly, we tested the  
18 compounds in combination as well. We did what we call the summer  
19 and the spring mixture. It turns out, all these compounds were  
20 supposedly applied in the spring, but only metolachlorine atrazine  
21 were still there in the summer, according to analysis we had done in

171

1 two different laboratories.

2 We tested each compound individually. We tested all 10  
3 compounds combined, at now several doses. We started out with one  
4 dose and then we tested metolachlorine atrazine together and we also  
5 tested bicep, which is the commercial metolachlorine atrazine mix,  
6 the two herbicides. Again, we color-coated everything.

7 I think you are looking at what, 30, 60, 90 cages, three  
8 replicates of all the treatments, times 30 animals per replicate.  
9 Everything was color-coated. Same endpoints -- we looked at time in  
10 metamorphosis, growth, development, size at metamorphosis, gonads  
11 and as each animal metamorphosed -- first, I want to give you a little  
12 bit about the rotation, because what you are looking at now are the  
13 tanks. Each has their own color-coated air hose, each has it's own net  
14 that is color-coated that's maintained in a plastic bag to avoid  
15 contamination. Everything is covered with a drop cloth when it's  
16 moved around. We have all our treatments and controls to analyze for  
17 contaminations as well. What you are looking at now, sort of a  
18 schematic of the different treatments and we do keep them in all in a  
19 row to avoid contamination and confusion. We also do a few other  
20 things.

21 What I'm showing you now is that every time we do a water

1 change, we rotate the tank so that no one tank is ever sitting in the  
2 same place at one time. I'm going to make tank number one white. If  
3 it was here, on Tuesday, Wednesday, Thursday, Friday -- on Friday it  
4 would end up here. Three days later it would end up here. So, we  
5 have a rotation pattern. We don't do it random. There are reasons for  
6 that if you want to talk about it. We also -- I designed where the  
7 tanks go. We put -- in this particular experiment, the reason these are  
8 white, is that we put controls on to the ends and in the middle. But  
9 they weren't always there of course, they were always being rotated.  
10 What this allowed us to do is test if there were position effects, front-  
11 to-back to test if there were effects from left-to-right.

12 And I organized this in such a way so that all the spring  
13 samples were blocked together and all the summer samples were  
14 blocked together. I did it rather than randomized it, because if there  
15 were a left light or a front back effect, particularly if there was a left-  
16 right effect, I could separate this into two experiments and have a  
17 control as a calibrator in the middle.

18 You now are just looking at time to metamorphosis and average  
19 time to metamorphosis for each of these tanks and all I'm doing is  
20 putting this here to tell you there is no difference between front and  
21 back or left and right.

1           In other words, no matter where these controls were -- and they  
2           were never static -- every three days they were moving. We keep  
3           maps of every time we do a tank change. What I'm telling you is that  
4           there is no position effect. So, we have controlled and tested for  
5           these things. There are also atrazine tanks where we can look for for  
6           atrazine by position effect or atrazine by position by tank, if you will.

7           So, we control for those things. The other thing we did was, as  
8           each animal metamorphosed and got that number that I told you about,  
9           each one of those 3000 animals were individually housed in a deli  
10          cup, so that we can monitor each individual's time and metamorphosis  
11          and size at metamorphosis.        For all 3000 individuals, we know  
12          when the four limbs emerged, we know how long it took it's tail to  
13          absorb and they were still maintained in treatment water. We know --  
14          we knew eventually what the sex was. We had data on individual  
15          animals that we followed from the time the four legs came out.

16          What I'm going to show you -- I'm going to break it down. We  
17          looked at the individual compounds, plus the mixtures and what I  
18          want to show you actually, it is quite interesting. No metalaxle  
19          wasn't so good for tadpoles, so we lost some data on the metalaxle.

20          No other compound seemed to affect metamorphosis  
21          significantly. No other single compound except atrazine had some

1 inhibitory effect on metamorphosis. I'll tell you about it.

2       What was more interesting was, if you look at controls, this is  
3 now number metamorphosing and this is day on the X-axis, here is the  
4 controls. That blue line is the average time to metamorphosis. The  
5 summer mixture is metolachlor atrazine (ph). There is a delay in  
6 metamorphosis. The spring is the ten mixtures -- ten compounds  
7 together -- there is an even greater delay in average time to  
8 metamorphosis.

9       What is interesting is it almost seems like -- this may spark  
10 some discussion, it almost seems like the tadpoles are somehow  
11 counting the number of chemicals they are exposed to. For example,  
12 this spring mixture of the ten compounds I showed you -- there is only  
13 .1 microgram per liter of each individual compound. So, there is only  
14 a total of one microgram per liter of pesticide. Again, it is ten things  
15 at .1.

16       Even if I give atrazine at 200 parts per billion, it won't produce  
17 this kind of effect. Each of those compounds at .1 part per billion  
18 won't produce and effects on metamorphosis, even atrazine at 200  
19 parts per billion won't produce this kind of effect, but when you put  
20 all compounds together, as low as .1 part per billion -- one part per  
21 billion kills them all. You get these kind of delays in metamorphosis,

1 which are greater than delays that you get with the two compounds.

2 What is more is Paula Case, who is actually here today, as well, has  
3 been looking at the thyroid glands.

4 If you look at the thyroid glands as controls compared to  
5 animals from these mixtures, you get large -- what appear to be  
6 enlarged thyroid glands. I don't have the quantitative data yet. We  
7 are still doing the histology. Like I said, it is thousands of animals.  
8 We're looking at thyroid gland size, volume, follicle size, colloid  
9 size, all those measures of thyroid inhibition and we're also, of  
10 course, looking at first to last animal per tank. We'll looking at  
11 trends in the thyroid gland within a tank and those things. But it  
12 appears -- I mean, certainly, there is statistically significant effect  
13 with these mixture on timed metamorphosis and it may be correlated  
14 with some type of effect on the thyroid gland that we're starting to  
15 look at.

16 One of the other things is, I would never have looked at the data  
17 this way, but Kelly Haston -- this is actually from a different  
18 experiment -- Kelly Haston has been analyzing some data. These are  
19 animals from Utah that she is looking at and what she has done is --  
20 here are controls and she has looked at rank order. She just looked at  
21 the first animal to metamorphosis and plotted the days to

1 metamorphosis, animal number 1, 2, bla, bla, bla.

2           What you will notice is, it seems that they start to accelerate in  
3 the last third on the tail. At .1 part per billion atrazine, you see the  
4 same acceleration. It seems like they start to slow out, to slow down  
5 when they are at the 25 parts per billion dose.

6           It seems and in the other data set which we'll go back to in just  
7 a minute, it seems that the inhibition of metamorphosis is due to the  
8 last third slowing down. The first two-thirds seem to do -- to be no  
9 different from the control. Does that make sense?

10           There is overall inhibition of metamorphosis. I'm going to  
11 show you the same thing with the mixtures. If you look at the first  
12 third of the animals, controls compared to pesticides, they are the  
13 same. There is no difference. If you look at the middle third, they  
14 start to slow down. If you look at the last third, they really start to  
15 slow down.

16           I don't know if that's because you're taking animals out of tank,  
17 they're now effectively at a higher dose because there is less animals  
18 in the tank to deal with the load or if it's just the slower ones are  
19 more susceptible to whatever effect the pesticide have.

20           Let me stop. Does that make sense?

21           The reason I bring it up is because there is a consequence to

1 this. You are now looking at time to TR, time to complete tier  
2 resorption (ph) in days. These are controls. In this case, it is nine  
3 replicates, because we're talking about the same experiment.

4 This is S. metolachlor, and that is atrazine. We're only looking  
5 at the last third of the animals. The first two-thirds come out -- there  
6 is no statistical significance difference. There is an overall  
7 difference, but it is because the last third are slower. If you look at  
8 controls, S. metolachlor atrazine alone delay metamorphosis by a  
9 week, almost ten days. Either one of the compounds.

10 If you combine the two compounds, however, metolachlor and  
11 atrazine mixed together at the same proportion that they would be  
12 mixed in bicep or bicep itself, there is a delay of about 20 days -- or  
13 about two weeks. Sorry.

14 When I first presented these data to some people in EPA, they  
15 asked, it is statistically significant, but is it biologically significant?  
16 Would two weeks make a real difference.

17 My best answer I like was one that I found when I was actually  
18 in Belize for something, but here is a cornfield. It turns out they have  
19 a leopard frog in Belize. I was supposed to be on vacation. There is  
20 my kids. They don't have anything to do with it.

21 There is a runoff pool or a pool here on the side of the road. If

1 you look in that pool, there are leopard frog tadpoles in the pool. The  
2 very next day, they are gone. So these are just dead tadpoles. That  
3 same pool that I showed you earlier has desiccated.

4 There are a lot of -- there is a great deal of data now that there  
5 are amphibians -- especially amphibians that breed in temporary pools  
6 that are adapted to pond drying and accelerated metamorphosis.

7 There has been a great deal of study including work by people  
8 on this panel to look at some of the hormones involved in that  
9 accelerated metamorphosis and involved in that response to pond  
10 desiccation.

11 What is the possibility that the evolution, the adaptation to  
12 pond desiccation and the hormones involved, thyroid hormone,  
13 potentially corticoids, what is the possibility that the most important  
14 thing about pond desiccation now is an increase in the concentration  
15 of the pesticides that are in that runoff, for example, such as the  
16 metolachlor and atrazine, which seem to be the only two persistent  
17 pesticides and which combined appear to inhibit metamorphosis?

18 I think there is a biological significance when you consider this  
19 adaptive response.

20 The other significance is the following. Part of this resistance I  
21 was telling you about is this. The first 25 percent of the animals in

1 several of the rana pipiens populations we look at have this  
2 arrangement at metamorphosis. So remember I showed you before the  
3 gonads were clearly differentiated, they had spermatids developing,  
4 they had oocytes in the ovaries?

5 Populations such as these in Nebraska and also Connecticut,  
6 one population we have shows this, the testis, we believe this is a  
7 testis, still has quite a bit of -- that's not just weak testis, quite a bit  
8 of cortex -- medulla in tact as well as the cortex, so it's relatively  
9 undifferentiated, and females from some of these populations almost  
10 look like a xenopus.

11 There is a single oocyte here and an ovarian vesical, but you  
12 don't see oocytes the way you see in some of these Wisconsin  
13 populations, some of the gonads that I showed you earlier.

14 So the hypothesis we are working at now is that these animals  
15 that metamorphose, these populations that metamorphose quickly, but  
16 have delayed gonadal development relative to their somatic  
17 development, may be resistant or may escape the effect because these  
18 gonads aren't going to differentiate until they are out of the water.  
19 These animals have already metamorphosed. Does that make sense?

20 If you look at the animals to metamorphose, those are the ones  
21 that tend to be more sex reversed or that tend to have the oocyte

1 statistic of the oocytes.

2 I guess the point I'm getting at is the more severe  
3 hermaphroditism or the more severe gonadal abnormalities tend to be  
4 in the animals that metamorphose last.

5 Of all the pesticides we looked at, that list I gave you of ten,  
6 the only one that produces the effects in our laboratory so far is  
7 atrazine.

8 Now what I'm telling you is that -- and I'm telling you not only  
9 does atrazine produce the effects, but the effects are more severe in  
10 the slower developing animals.

11 And now I'm telling that you when you mix atrazine with other  
12 compounds, and in this case metolachlor, you get delayed  
13 metamorphosis in the last animals to metamorphose. When atrazine is  
14 mixed with other compounds, there is -- I don't want to use the word  
15 synergism because -- there is an enhanced effect because essentially  
16 duration of exposure has been increased.

17 Again, by going into the field, there is value. There is  
18 uncertainties, but those uncertainties have allowed us to design a  
19 more realistic, yet controlled experiment in the laboratory.

20 What you are looking at now is another consequence of -- here  
21 are controls, the summer mixture, which is just the two compounds,

1 metolachlor and atrazine. The spring mixture, which is the ten  
2 compounds. You are looking at body weight and snout vent length,  
3 and on the X axis, time to metamorphosis. This is going to come up a  
4 couple times.

5 In other words, what you are looking at is you are looking at  
6 what it really means to be a tadpole. We talked about that little pin  
7 size egg with no yolk sack in the beginning. The point of being a  
8 tadpole is to get big enough so that you can metamorphose and be an  
9 insect or a carnivore. It's a growth period.

10 And so the longer you take, if you look at controls, the longer  
11 you take to metamorphose, the bigger you should be. In other words,  
12 if you metamorphose quickly you have had a shorter growth period, so  
13 you come out smaller. Whereas, you know, your brother, which takes  
14 longer to metamorphose or sister which takes longer to metamorphose  
15 is going to be larger because they have had a longer growth period on  
16 average.

17 If you mix these two compounds together, you start to reverse  
18 that trend. If you mix ten compounds together, you start to reverse it  
19 even more.

20 So there are consequences of not just individual compounds and  
21 the effects on the gonads, but there are also consequences when those

1 compounds are given in a more realistic environment, i.e., one of  
2 these chemical mixtures.

3 One of the other consequences especially with the ten  
4 compound mixture, although we see this a little bit with atrazine and  
5 metolachlor, here is a control at metamorphosis, a healthy animal.  
6 This is an animal exposed to the ten compound or the spring mixture.  
7 And you can see he doesn't look happy and he can't walk straight.

8 Apparently, there is some immuno compromise when the  
9 animals are exposed to this mixture of ten chemicals. We saw it very  
10 low frequency with the metolachlor and atrazine.

11 But what is happening is this animal has a microbacterium.  
12 Apparently they all have it. It's a symbion. But they don't succumb  
13 to this inner ear infection unless they are immuno compromised. And  
14 now we're at thymus and trying to do some immuno challenges to look  
15 at, try and characterize that effect. But it is another effect, again, of  
16 the combined chemicals and something we should consider.

17 So we have done these controlled studies. Used that to find  
18 endpoints that we then used to do field studies. Maybe the most  
19 valuable thing about the field -- certainly one of the most valuable  
20 things is we were able to use the information we got in the field to set  
21 up some real simulations in the laboratory where we can do more

183

1 realistic exposures with atrazine and its companions.

2       The last thing we have done, I won't be able to give you data  
3 yet because the work is still in process, is we really brought the field  
4 home.

5       We collected, I'm not going to even try to say the number, but  
6 hundreds of -- does anybody remember how much? A lot of water.  
7 We collected it from several sites. Sites that we expected to be  
8 contaminated, sites that we knew had high incidence of  
9 hermaphroditism.

10       Temporally stored it while we were on the road just overnight.  
11 Then we transported it back to Berkeley in an 18-wheeler truck with it  
12 all maintained frozen. We had somebody freeze Wholefred (ph), a  
13 solution, back at the lab so that we have -- Wholefred is just a  
14 solution we use for rearing controls. So we have control water frozen  
15 back at the lab too.

16       The reason for that is to answer the question that I think all  
17 critics are going to ask. And that is what if populations just vary?  
18 What if it's really just that animals, frogs from some of these sites in  
19 Nebraska, just like their gonadal dysgenesis and testicular oocytes,  
20 that is just how they are, and animals from Utah that aren't interested  
21 in that?

1           What we can do is and what we have been doing is each week  
2 we take out a bucket of water, we can raise Utah animals in Utah  
3 water and expect them to be normal. And if it's really just population  
4 variation, animals transplanted from sites like in Nebraska and  
5 Wyoming, for example, will still come out intersexed even if they're  
6 in that Utah water.

7           Whereas if you raise them in Nebraska water, if it is just normal  
8 population variation, the Nebraska water won't affect the Utah  
9 animals.

10           The alternative hypothesis, of course, is it's the water and we  
11 know the chemical -- what chemical contaminants are in the water.  
12 We measure it over time to make sure it's stable. USGS has been  
13 doing that for us.

14           If it's the water, no matter where you come from, Nebraska and  
15 Wyoming water will induce the hermaphroditism. And that's ongoing.

16           We also have Wholefreders controls for everybody that we're  
17 using as well, and we'll be bringing back, helicoptering water out of  
18 Montana to do similar things with the water there.

19           So you know, the real question, I think is, I think both the field  
20 and the laboratory studies are important. I have listened to groups  
21 argue about the relative importance and what should be done first.

1 I think it was the last group that was up said or maybe it was  
2 someone on the panel said maybe you do both at the same time.

3 I think it is important to do both at the same time so that you  
4 can characterize what is natural for the individual populations,  
5 characterize what is happening in the field, and try to simulate and  
6 characterize what they are exposed to as well as maybe bringing water  
7 back and doing the kinds of things where you can really have -- as  
8 close to as you can get as having a field in the lab where you can  
9 control everything and really determine if it's really the water that  
10 makes things like this and which compounds in the water.

11 So that's the sort of lab field, lab model that we have been  
12 working on for the last three years. I don't know if I should pause  
13 here for questions. Because the next thing I want to do is really  
14 address this cause and effect question. Some of the questions that the  
15 panel is charged with about dose response, et cetera and mechanism.

16 DR. ROBERTS: Let's go ahead and ask the panel if they have  
17 any questions on anything you have presented so far.

18 DR. HAYES: Yes, please.

19 DR. ROBERTS: Dr. Skelly?

20 DR. SKELLY: I have some questions about your field methods  
21 which pertain to the Nature and EHP papers. I guess they are

1 important because you are continuing to use these field sites.

2 DR. HAYES: Yes.

3 DR. SKELLY: I guess I'm curious how you selected those  
4 eight locations? You talked about it briefly, but I would be interested  
5 in some more details. And then for those general locations how you  
6 selected specific wetlands.

7 And then how you -- you said you collected 100 animals. I  
8 think Dr. Ashby was referring to -- the next couple sentences in there  
9 said you analyzed 20 of them. Maybe you can clarify that. I think  
10 that's what he was referring to.

11 But how did you actually collect those animals? And  
12 specifically, you said you were targeting metamorphs and you were  
13 basing that on size. Were you identifying the metamorphs before you  
14 picked them up or were you doing that afterwards? And also -- well,  
15 that's enough for now. Why don't you chew on that a little.

16 DR. HAYES: Let me back up for a second. It might be useful  
17 to have this out.

18 The first part of the question is how did we pick the sites. One  
19 is I looked at this map, and I made some predictions about what is  
20 going to be a reference or what we call a control site. What is going  
21 to be a reference site. What is going to be a contaminated site.

1 I based my hypotheses on what was reference and what was  
2 contaminated based on this map which was on sales.

3 DR. SKELLY: Did you sit there in Berkeley before you got on  
4 the road and just picked counties and picked like eight counties and a  
5 set of back-ups?

6 DR. HAYES: I picked the site in Utah in part because I was  
7 working with somebody in Utah Fish and Wildlife who knew where  
8 there were healthy rana populations. And those were animals that we  
9 already had in a colony in a laboratory.

10 And then because I was using one in Utah I wanted a nearby  
11 site that would likely be contaminated. So I went to this county  
12 which, I believe, is Cash County, because there is golf courses and  
13 cherry growing there. So I wanted sort of paired sites.

14 I picked everything along a parallel. I mean, I'm making a joke  
15 about I 80, but I picked it on a parallel because of the differences in  
16 the development times as you go north south.

17 DR. SKELLY: (Inaudible) You got an altitude; no gradient  
18 there, though, too. Right?

19 DR. HAYES: Yes. We have all that in the paper and the GPS.  
20 Yes, you are correct.

21 But we recorded that and took -- originally, we were going to

1 collect in Nevada, for example. There are just no viable populations  
2 there and the state wasn't going to give us a permit to collect 100 from  
3 any site.

4 We did get some animals -- there was die off here or  
5 something, but we haven't analyzed them and, of course, there was a  
6 die off on Native American land. Somebody was working there, but  
7 we didn't use any of that. So the site from Utah was where we  
8 knew healthy populations, then we chose a site -- sorry it's hard see  
9 from way back here, we chose a site where there might be  
10 contamination.

11 Wyoming we picked as a reference site because we thought  
12 there would be no use in Wyoming. That that would be a good site for  
13 a reference.

14 In Nebraska I chose Cherry County because it was a reference  
15 site surrounded by atrazine. I knew I could find -- paired as best you  
16 can find contaminated samples or contaminated sites in Nebraska.  
17 Then I wanted several sites in Iowa, Illinois, and Indiana.

18 In Iowa I worked with somebody from the university who got us  
19 permission to go on the farm lands, because we wanted some runoff.  
20 He got us permission. Fred Jasen is his name. I think he is  
21 acknowledged in the paper -- got us permission to go into the wildlife

1 refuge.

2 So we had within a contaminated state, if you will, we had  
3 cornfields where we expected it to be high and then we had protected  
4 areas where they weren't using atrazine on that site because it's a  
5 wildlife refuge.

6 Then in Illinois and Indiana, we were given permits, but we  
7 never found a site we were comfortable enough that there were enough  
8 frogs that we felt like we could take 100 and be -- not ethical, but we  
9 just didn't feel like there was enough animals that we could do it.

10 The sites, in general, the counties were chosen a priori,  
11 because, in fact, when you write for the collecting permit you have to  
12 list exactly what counties you are going to. They were chosen a priori  
13 the sites. My students can tell you, we drive around until we find  
14 frogs.

15 So that specific sites in places we had no knowledge, like in  
16 Iowa where somebody helped us, we just drove around and went recon  
17 until we found frogs.

18 DR. SKELLY: So you didn't use like National Wetland  
19 Inventory maps, or anything like that?

20 DR. HAYES: No. I did not. No.

21 DR. SKELLY: And you didn't put, say, all the counties along

1 I-80 into a hopper, stratify them by atrazine and then select?

2 DR. HAYES: Like in Wyoming, we drove until we saw water.  
3 We would get out and walk and look for frogs. It's the same way I  
4 hunt in Lake Victoria in Africa.

5 In terms of picking the metamorphs, at each site with the  
6 exception of Cherry County, at each site we found a sample of animals  
7 which we have preserved as a reference that still had tailbud. So we  
8 knew they were newly metamorphosed. We used that snout vent  
9 length as our basis for choosing who was a new metamorph and not.

10 DR. SKELLY: So that is going to vary among sites, though --

11 DR. HAYES: That varies greatly among sites. Yes. The size at  
12 metamorphosis ranges in Nebraska from the place in the cornfield in  
13 Nebraska to about the size of my thumb to -- where is a big site?  
14 Some sites they came out this big.

15 DR. SKELLY: You were doing this in latter July, I guess?

16 DR. HAYES: Yes, because I was there for my birthday. It's  
17 July 29th. So it was in July, yes.

18 DR. SKELLY: Did you know that you were hitting the same  
19 point in the metamorphic period for all of these different sites?

20 DR. HAYES: I can't -- no, I can't know that. And I know --  
21 what I can tell -- the reason I said except for Cherry County,

1 Nebraska, what I can tell you is that at each site we collected a subset  
2 of animals that still had small tail remaining. You know, sort of  
3 stage, whatever, 43, 44, 45, except Cherry County where we could not  
4 find any.

5 And there is some indication in Cherry County that they might  
6 even overwinter and that it might be different aged animals.

7 DR. SKELLY: I'm sorry I asked a long question, but how did  
8 you collect them, specifically?

9 DR. HAYES: Depends on where we were. So on North Plat  
10 River, we walked around with hand nets and dip nets and caught them  
11 along the river.

12 In Cherry County we unsuccessfully dug pitfall traps, and we  
13 ended up -- in fact, by the picture, we ended up trapping them in the  
14 grass with the same net.

15 In the golf course we walked, we could walk along the water  
16 and they would hop up and rush on land and catch them by hand.

17 In other places we walked along with nets. It depended on the  
18 terrain. And the animals behave very differently if they are in a river  
19 versus a pond versus a flooded meadow.

20 DR. ROBERTS: Dr. Heeringa.

21 DR. HEERINGA: I have a question about the lab field

1 simulation where you actually look at the mixtures of compounds.  
2 And as I look at the control in spring, spring summer graphs there  
3 clearly at least by the number of points on these graphs is substantial  
4 mortality differential across those which -- is that the case?

5 DR. HAYES: Let's pull it up. For the ten mixture, yes. And --  
6 that would be the spring mixture.

7 For the summer, I don't recall that there is any difference in  
8 mortality. And we suspect, in fact, that the problem in the spring  
9 mixture is the metalaxyl (ph). That if we pull the metalaxyl out of the  
10 spring mixture, we can mix the nine compounds. Because metalaxyl  
11 doesn't do so well for the animals.

12 DR. HEERINGA: It was metolachlor that is part of the summer  
13 mixture?

14 DR. HAYES: The summer mixture is atrazine plus  
15 metolachlor. And the spring mixture is all ten of those compounds,  
16 tebupirimphos, cyfluthrin, the whole thing.

17 DR. HEERINGA: Since you have timed data here have you  
18 looked at all at applying a survival analysis technique to that data  
19 which would account for the censoring?

20 If you knew when these tadpoles died, you do know that at that  
21 point?

1 DR. HAYES: I know exactly what happened to each individual  
2 of these 3000.

3 DR. HEERINGA: Have you considered or have you done that,  
4 applied a survival analysis method that would adjust -- your endpoint  
5 that you are looking at is time to metamorphosis which is the event of  
6 interest here. But there is a competing risk which is the mortality of  
7 these individuals. And if the two were confounded in that competing  
8 risk, I think it could change the interpretation just as a lot of other  
9 human survival or event studies have.

10 DR. HAYES: I have not done such a thing on this data set.  
11 This is an unpublished data -- do you have a copy of it? Is that what  
12 you are looking at?

13 DR. HEERINGA: No.

14 DR. HAYES: I thought you were saying you had a copy. This  
15 is an unpublished data set. This is rana pipiens. We have repeated  
16 this now with xenopus laevis looking at multiple doses and things like  
17 that, but the analysis that you are mentioning is not something I have  
18 done thus far.

19 DR. HEERINGA: Just a comment, it would be a very valuable  
20 thing to add to the analysis of this particular data since you have all  
21 the time dependent measures and you know the fates of these

1 individuals, either death or survival and survival time to  
2 metamorphosis, so I recommend it. DR. HAYES: I  
3 might certainly talk with you more about that. Because I am not  
4 familiar with the type of analysis you are talking about, to be honest.  
5 But it sounds like something we would be interested in doing.

6 DR. ROBERTS: Dr. Kloas and Dr. Denver.

7 DR. KLOAS: To the presentater, I would like to keep on with  
8 the inhibition of metamorphosis. In rana pipiens you find something  
9 of delay on metamorphosis by atrazine. In xenopus not. Do you have  
10 any explanation for that?

11 DR. HAYES: There are some data, not my data that I can show  
12 you, that suggest that atrazine inhibits metamorphosis in xenopus.

13 Rana pipiens, the true rana pipiens vary incredibly. So even  
14 some populations of rana pipiens we get delays in metamorphosis and  
15 some we don't. I can them tell you why. I think why anyway.

16 So for example rana pipiens from Wisconsin, I can pull the data  
17 up, I actually have it here, don't show any delay this metamorphosis.  
18 But rana pipiens from Connecticut take about two months to  
19 metamorphose. Rana pipiens from Wisconsin, same room, same  
20 temperature, same tanks take about five months.

21 So there is such a larger variation that I think you don't pick up

1 the effect. So I don't know if it is a real biological effect that it  
2 occurs in some populations and not others but just the nature of the  
3 difference in time to metamorphosis. But treated the same way, they  
4 are very different size, very different time to metamorphosis.  
5 So it depends on the population, even with rana pipiens. It doesn't  
6 surprise me that xenopus and rana pipiens might respond differently.

7 DR. ROBERTS: Dr. Denver?

8 DR. DENVER: I have just a general point of clarification. I  
9 just wonder if your data set is more robust than what is actually  
10 presented in the public literature. Because you mentioned a number  
11 of times that you have 10,000 and more observations over the course  
12 of five or six years. And --

13 DR. HAYES: Sorry. That's not all atrazine, though. We do --

14 DR. DENVER: Okay. But assuming you have a subset of that  
15 that is atrazine, are there more data than what is presented in the  
16 public literature that can be made available?

17 DR. HAYES: Yes. You will see more of that today.

18 So for example, some of the data involved a big experiment we  
19 did to look at testosterone and estrogen levels in the larvae, and we  
20 were unsuccessful. I know Dr. Kelly has measured it. I have  
21 published and measured on steroids. We were unable to measure

1 steroids in the developing larvae in response to atrazine in the  
2 controls or atrazine treated.

3 So some of the observations were that study, which hasn't been  
4 published.

5 Some of them were some work we have done with the  
6 antiandrogen, suproacetate (ph) which I will present a little of that  
7 today. Some of them were with the ongoing study, our growout study  
8 where we are still maintaining the animals and taking blood samples  
9 in the adults.

10 DR. DENVER: Do you recall the sample size in the  
11 testosterone measurements in the PNAS paper? I just, I couldn't find  
12 it.

13 DR. HAYES: Melissa, do you know? Four controls and four --  
14 the sample size in the testosterone measurements in the PNAS paper?  
15 How many animals per treatment?

16 Four individually housed animals. Yes, they were individually  
17 housed. So in this case the number of animals and the number of  
18 replicates are the same per treatment.

19 DR. ROBERTS: Dr. Coats, Dr. Kelly, Dr. Green.

20 DR. COATS: I have a question about the laboratory studies.  
21 Your paper says that the doses were confirmed by outside labs. What

197

1 does -- how does that happen?

2 DR. HAYES: We sent blind samples. Some of them, for  
3 example, went along with our samples from the field. We sent  
4 samples from the stock that we make. We sent samples from day zero,  
5 and then we also took samples at the end of three days when we  
6 changed the water.

7 What went in on day one and then we changed the water every  
8 three days and what came out, we took samples from all replicates  
9 across all doses. That was done -- when I was on the Ecorisk panel,  
10 that was done by PTRL West.

11 In the subsequent study PTRL West also did our sampling. And  
12 then in the rana pipiens study and some of the xenopus study it was  
13 done in triplicate by Iowa Hygenics, PTRL West, and by USGS,  
14 William Battaglin.

15 But now we are solely working with William Battaglin and  
16 USGS, because the data are the same from all three laboratories. All  
17 except that one sample that I talked to you about.

18 DR. ISOM: Did they look for any metabolites over the three  
19 days that might have been --

20 DR. HAYES: Yes. Those are published in the EHP paper.

21 De-ethyl and de-amino atrazine and one other. And in the field

1 samples they looked for other triazines as well.

2 DR. COATS: Did they show any -- I didn't see anything about  
3 the metabolites. How much they were in the lab studies.

4 DR. HAYES: Oh, in the lab studies.

5 DR. COATS: Yes.

6 DR. HAYES: I do have those data from the lab studies as well.  
7 They are published in the EHP paper for the field studies, but they  
8 also send data on the metabolites in the lab studies.

9 I don't know off the top of my head what they are. Atrazine  
10 over the course of three days decreases by 30 percent. But I don't  
11 remember what the major metabolites are over the three days.

12 DR. COATS: Do you think the metabolites could have any part  
13 of the activity we have seen?

14 DR. HAYES: I would love to -- in fact, I have written to  
15 Syngenta to ask for samples of the metabolites to test. I think it is  
16 possible.

17 We have tested, I don't have the data, because we're still doing  
18 the study. But we have tested nine other triazines and two species. I  
19 would love to add metabolites to one of those studies, but we don't  
20 have access to them.

21 DR. COATS: Do you have any body burden data on the frogs

199

1 from the lab studies or the field studies?

2 DR. HAYES: I do not. My recollection, when I was on the  
3 Ecorisk panel, is that it wasn't done because there was literature, I  
4 think, on bull frogs, you might have to ask Allen or Ron, that there  
5 was data on bull frogs that basically showed it didn't bioaccumulate  
6 and that it was water soluble and that was negative in the tadpoles, my  
7 recollection.

8 And that's why we chose not to do it in the Ecorisk panel. We  
9 froze tadpoles actually for the analysis, but it was decided we  
10 wouldn't do the analysis when I was on the panel. It is not something  
11 that I tried to do independently. No.

12 DR. COATS: And you didn't do them in the lab studies either  
13 then?

14 DR. HAYES: No, oh you mean body burdens from the field?  
15 No, I didn't even consider it, and we didn't do it in the lab either.

16 DR. COATS: Okay.

17 DR. HAYES: Only the water.

18 DR. COATS: Okay.

19 DR. HAYES: And we also had food samples that went to PTRL  
20 West or the food dissolved in the water.

21 DR. ROBERTS: Dr. Kelly.

1 DR. KELLEY: This is following up on categorization of the  
2 kinds of gonads that you get with exposure to various substances.  
3 And you use the word hermaphrodite in describing the field data.

4 But my understanding is that your conceptual scheme for  
5 normal development of the male gonad in rana is that they start out  
6 with a distal segment that has, how can I say, female potential that  
7 may, in fact, contain oocytes. And then during maturation that part  
8 of the gonad disappears and the gonad shortens.

9 So when you have an animal that has frank oocytes and  
10 maintenance of that distal segment, what you are getting is failure of  
11 differentiation of the male gonad that's maintaining this more female  
12 like part. Right?

13 DR. HAYES: Uh-huh (Affirmative).

14 DR. KELLEY: So we might begin to think about a different  
15 word for describing that.

16 DR. HAYES: No, I agree. I should be more careful. I think  
17 hermaphrodite is inappropriate for what we find in rana.

18 DR. KELLEY: And what do you think about the word intersex?  
19 These words, you know, carry connotations.

20 DR. HAYES: Intersex, in my recollection, historically has been  
21 used interchangeably with hermaphrodite. I would be reluctant to use

201

1 the word intersex.

2 DR. KELLEY: Is it possible that many of the effects you see  
3 are due to developmental retardation of some sort?

4 So it maybe, in fact, that's part of your hypothesis for how they  
5 escape atrazine effects. So if you keep the posterior part of the  
6 gonad, it is available to plump up if it gets any estrogen and becomes  
7 vitollegenic.

8 DR. HAYES: Yes.

9 DR. KELLEY: Right. So that's possible. And you don't -- you  
10 tried, I'm confused about this. But you have tried to breed some of  
11 these animals?

12 From the original study you didn't have enough animals left to  
13 breed. I guess that was the xenopus study.

14 But from these rana animals, do we have any data on what the  
15 fertility of an animal with a maintained oocytes and good testicular  
16 tissue would be?

17 DR. HAYES: We have from the xenopus, yes, we have tried  
18 multiple times to breed the xenopus. Like I said -- and correct me if  
19 I'm wrong, but my experience is that the males become sexually  
20 mature earlier and at a smaller size and then the females take a little  
21 bit longer.

1           And we have tried to breed within that, you know, not to bring  
2           in other animals to breed with our treated animals, but often what we  
3           think are the little males, as I said earlier, eventually we will inject  
4           them and try to get them to breed and they'll lay eggs.

5           The problem is you can't prove that they used to be males. You  
6           just now know you have a preponderance of females, so, in xenopus.

7           In rana now we do have a colony that is -- we actually have  
8           atrazine treated, Nebraska water treated, Wyoming water, they have  
9           been raised in a whole bunch of different waters. And they are now  
10          adult size. And we have blood samples and we are starting to measure  
11          hormones, but we haven't attempted any breeding.

12          And as you know, rana is a tempered species, will be a little  
13          more difficult.

14          DR. KELLEY: And Witchie in his early studies of estrogen  
15          treatment, partial estrogenization of xenopus pointed out that  
16          although at early stages you had ovaries that were -- you had gonads  
17          that were -- contained both testes and ovarian tissue, which is very  
18          unusual.

19          At later stages it looked to him like some internal regulatory  
20          process happened and the ovarian tissue actually went away.

21          And I wondered if this was also true with the animals that you

1 had that were atrazine treated. In your older animals, do you have a  
2 lower incidence of frank ovarian tissue or does it persist?

3 DR. HAYES: In the case of xenopus, again, I think that they  
4 actually completely become females. But I can't prove that. So we  
5 have a preponderance of females. We don't -- well, that's based on  
6 external and on egg laying. These are animals that are still alive. So  
7 again I can't -- they may hermaphroditic when we look inside.

8 For the rana we have not -- we have grown those up. I don't  
9 think we have opened any of those -- I don't think we have euthinized  
10 any to look at them. So we have ones that are now -- gosh they are a  
11 year old now. I have no idea what they look like inside.

12 DR. KELLEY: Here is my suggestion, which comes originally  
13 from Witchie who is no longer with us, I believe. He pointed out that  
14 if you raise -- he got complete sex conversion and then back crossed  
15 the animals when we raised groups of tadpoles in estradiol.

16 Some of those back crossed animals when mated with males  
17 gave rise to entirely male offspring.

18 His interpretation of those data was that those were feminized  
19 ZZ individuals that normally would have been male.

20 DR. HAYES: We have done that also.

21 DR. KELLEY: So you have ZZs around.

1 DR. HAYES: Estrogen treated, yes.

2 DR. KELLEY: So my suggestion would be since you know that  
3 a ZZ individual will normally -- 100 percent of the time, at least from  
4 his data, go on to become male, you know in advance of the  
5 interpretation of whether it is a real female or an estrogenized female  
6 becomes much simpler.

7 And he suggested that as a major advantage of the system in  
8 the 50s and 60s when he was working on it. So my suggestion is that  
9 that animal is a more appropriate animal.

10 DR. HAYES: We have a huge ZZ colony that we have already  
11 screened and figured out who is a ZZ female and who is just a ZW --  
12 so we have that already.

13 DR. KELLEY: So you have that.

14 DR. HAYES: As a matter of fact, those are the animals that --  
15 my post doc who was doing the molecular work to look at Cyp 19 and  
16 all these things, those are the animals that she is using to do that  
17 work. So we have already started that.

18 DR. KELLEY: Thank you.

19 DR. ROBERTS: Dr. Green, Dr. Kloas, Dr. Skelley.

20 DR. GREEN: Could we look at that slide again with that rather  
21 sickly looking frog that you collected from one of the ponds that was

205

1 atrazine contaminated?

2 Did you actually culture microbacterium species from this frog?

3 DR. HAYES: I did not culture it, but John Parker, who is the  
4 veterinarian at our university did it. I misspoke. It's not an inner ear.  
5 It's microbacterium induced meningitis.

6 DR. GREEN: I think that's an important observation. I'm sure  
7 you are aware a couple months ago a Canadian group published a  
8 couple papers linking atrazine amongst 13 or 15 other chemicals as  
9 being associated with immunosuppression in wild caught rana pipiens  
10 and enhancing the virulence of a very common lung pathogen in rana  
11 pipiens.

12 I would encourage if you have these kind of specimens  
13 collected from the field to get a postmortem exam and look for  
14 granulomatous lesions of both microbacterium and count the number  
15 of parasites. Those two things together will significantly shorten the  
16 lifespan of these wild caught frogs.

17 This has implications beyond the effects on the gonadal  
18 development.

19 One other minor point, at least in the laboratory animal  
20 environment, we don't consider a marinum (ph) species to be a  
21 symbiotic species with an aquatic animal.

1 DR. HAYES: Sorry. I misspoke.

2 DR. GREEN: It is actually an opportunistic pathogen.

3 It is of great interest, but something I would strongly encourage  
4 you to pursue.

5 DR. HAYES: Sorry. I used the wrong word. By symbion, I  
6 meant that what John Parker, the veterinarian that I'm working with,  
7 has shown is that all these animals have the pathogen. But it is only  
8 showing up in -- I misspoke, regular red. Now we have to change the  
9 color code for the mixture.

10 These are the only ones that actually show the disease. I didn't  
11 put in my presentation, but we have a huge data site on parasite loads  
12 of the liver and the kidneys. I have it here with me, but --

13 DR. GREEN: I think that might be as important as the gonadal  
14 effects of herbicides and pesticides in the end.

15 T lymphocyte function is something that would be useful if you  
16 have blood samples on these animals when they are alive and are able  
17 to do it. I realize it is complicated in the field.

18 I know John Parker quite well and his work and I'm aware. And  
19 as far as I know, the microbacterium species hasn't been speciated,  
20 which takes time and complicated PCR. It needs to be validated by  
21 several laboratories.

1           So it will be interesting to see if this is marinum or many of the  
2 other possible species.

3           DR. HAYES: He has joined my laboratory for his Ph.D.

4           DR. GREEN: I didn't know that.

5           DR. ROBERTS: Dr. Kloas, then Dr. Skelly.

6           DR. KLOAS: I would also, Tyrone, like to come back to the  
7 PNAS paper.

8           First question that arises, if you are honing on the aromatase  
9 hypothesis, why didn't you measure estradiol?

10          I'm quite sure you did it and you tried it at least in parallel.

11          DR. HAYES: I have been unable to detect estradiol in the  
12 blood of adults -- or Melissa has been unable, I should say. We can  
13 detect it in females and we were unable to detect it in the larvae.

14          We're looking at Cyp 19 now. And we are a little better at our  
15 tritiated water assays. But we have been unable to detect it. We have  
16 tried to measure it.

17          DR. KLOAS: You were successful sometime -- but I know  
18 there is a lot of difficulties, but --

19          DR. HAYES: I've done it before too, but --

20          DR. KLOAS: I'm just referring to the adult samples.

21          DR. HAYES: Oh, in the adults?

1 DR. KLOAS: Your test showed up testosterone. Next question  
2 is with a radioimmunoassay you tried to detect it.

3 Could you discriminate between testosterone and  
4 dehydrotestosterone?

5 DR. HAYES: In the work that we have done, we did not do  
6 chromatography to separate testosterone DHT. We used -- I can tell  
7 you the specificity of the antibody, but of course there is some cross  
8 reactivity with DHT. I don't know it off the top of --

9 DR. KLOAS: So it's androgens in general probably.

10 DR. HAYES: Yes. That's correct.

11 DR. HAYES: So if you couldn't measure estradiol at the same  
12 time, which would substantiate aromatase hypothesis? I think for me,  
13 because testosterone or androgens are much more pronounced, so in  
14 estrogens in females as in males, of course. So it looks more rather  
15 like an inhibition of steroidogenesis.

16 DR. HAYES: I'm going to address that later. We did measure  
17 for estradiol. In fact, I think we extracted rather large volumes of  
18 plasma. We were unable to detect and have never detected estradiol  
19 in the plasma of males. We have tried.

20 DR. KLOAS: I'm sorry for that. It should be present --

21 DR. HAYES: We can find it in females.

1 DR. KLOAS: At least it should be measurable.

2 Next point, concerning --

3 DR. HAYES: Wait. Sorry. That's correct, right, Melissa?

4 Yes.

5 DR. KLOAS: -- the abnormalities you are referring to for -- you  
6 are covering everything. I think the biggest amount of abnormalities  
7 you showed up here, which is not -- which could not be seen in this  
8 PNAS paper is that you have unpigmented ovaries.

9 If you put unpigmented ovaries to the females, then you would  
10 have more or less a tendency towards feminization rather than  
11 demasculinization.

12 What do you think about that?

13 DR. HAYES: I will address that. I have some more data.

14 DR. KLOAS: Thank you.

15 DR. ROBERTS: Dr. Skelly and then Dr. LeBlanc.

16 DR. SKELLY: Just a couple -- one quick question first. Were  
17 you meaning to suggest based on when you were talking about Belize,  
18 that leopard frogs in North America live in temporary wetlands?

19 DR. HAYES: Sorry. Say again.

20 DR. SKELLY: You were suggesting does two weeks matter.

21 You showed the picture of your kids in Belize and everything. And

210

1 you showed the leopard frog tadpoles. Were you suggesting that  
2 leopard frogs live in temporary wetlands in North America?

3 DR. HAYES: In North America, we do find leopard frogs in  
4 temporary -- yes. For example, the little runoff corn ditches and  
5 things like that are not permanent standing water.

6 And in other sites. There are other noncorn runoff, but we find  
7 them in little pools that dry down. But I also used that as a general  
8 example for other amphibians that would be in temporary situations as  
9 well.

10 DR. SKELLY: The main thing I wanted to ask is have you, are  
11 you or will you be measuring fertility effects and male and female  
12 breeding behavior of lab reared and wild caught animals across  
13 atrazine gradients?

14 DR. HAYES: Boy, I would like to. I think for the rana pipiens  
15 we have a big enough colony of different animals that we can get  
16 animals year-round. But could we get enough animals breeding in a  
17 big enough sample size that we can assess that, I don't know.

18 I would like to. Like I said, we have a colony that's been either  
19 reared in some of this frozen water that we brought back or reared in  
20 atrazine or reared in atrazine plus metolachlor. They are a year old  
21 now. They are big. But whether or not they will actually take to the

211

1 laboratory and breed, I don't know.

2 DR. SKELLY: What about the field research program, your  
3 field research?

4 DR. HAYES: Will I try to assess --

5 DR. SKELLY: Fertility effects, breeding behavior.

6 DR. HAYES: For this season, our main goal is to truly identify  
7 sites that are uncontaminated and to identify sites where the incidence  
8 of, if such thing exists, the incidence of hermaphroditism are low so  
9 we can really have control or real reference sites.

10 Because right now the only site we have is the Utah site.

11 DR. ROBERTS: Dr. LeBlanc, then Dr. Gibbs.

12 DR. LEBLANC: I would like to revisit the field study where  
13 you look at eight different sites at various locations across the U.S.

14 When I first read that, the EHP paper, I couldn't for the life of  
15 me discern any relationship between atrazine levels and gonadal  
16 abnormalities.

17 And if I heard you correctly in your presentation, though, you  
18 made some generalization that, at four sites where atrazine levels  
19 were expected to be higher, the incidence of abnormalities were  
20 higher or something like that.

21 DR. HAYES: No. All I can say is where there is atrazine there

1 is hermaphrodites. There is no -- it is not--

2 DR. LEBLANC: But there was atrazine everywhere. Wasn't  
3 there?

4 DR. HAYES: No. There is one site where there was no  
5 atrazine. And there's one site where there's no hermaphrodites. All I  
6 can say is that's the only atrazine-free site we can find. And it  
7 happened they're the only site with no hermaphrodites -- or no  
8 testicular ovigenesis.

9 DR. LEBLANC: And then you did have one site where two of  
10 the analyses said there was no atrazine and one said there was very  
11 little.

12 DR. HAYES: That's right.

13 DR. LEBLANC: So there is really no atrazine there either, but  
14 because of the discrepancy, you didn't consider that one.

15 DR. ROBERTS: Dr. Gibbs.

16 DR. GIBBS: In trying to count for some of the discrepancies in  
17 the laboratory results reported by the various research groups, I have  
18 been struck by the small numbers of individuals used to found the  
19 experimental populations.

20 For example, in your PNAS paper basically you're working with  
21 the offspring from three pairs.

1 DR. HAYES: That's right.

2 DR. GIBBS: I'm not an amphibian toxicologist. Is that  
3 standard laboratory practice?

4 DR. HAYES: It is in my laboratory that we use a mixture of  
5 three animals. We count them out 5, 5, 5, 5, 5 until all the tanks are  
6 filled. And we use three per.

7 We also do natural breeds and track those animals so that -- in  
8 fact, I know this came up in the white paper, so that I can actually  
9 give you siblings of the animals that I used in the Syngenta study if  
10 you wanted to use those same animals.

11 But we typically do three or four pairs per experiment.

12 DR. GIBBS: My concern is that in one of the figures you have  
13 accounted for a difference in the magnitude of the results, not the  
14 effect, but as a population difference or a stock difference.

15 I'm just wondering how widespread those variations are due to  
16 really found our effects or -- of the individuals used might account for  
17 some of the discrepancies among the results of the different groups.

18 I'm curious if in your perspective that holds any water, that  
19 perspective.

20 DR. HAYES: I'll account for most of the discrepancies in the  
21 next part of my talk. I'm going to address when we talk of cause and

1 effect the source of much of the inconsistency.

2 Well, let me back up. Could there be "strain" differences?

3 Yes. There could be.

4 Do I think that there strain differences are the primary reason  
5 that we have discrepancies between laboratory studies that we are  
6 talking about now? No. The information I have suggests that there are  
7 other things that have confounded those data and that there are also  
8 perhaps some missing data.

9 DR. GIBBS: Thank you.

10 DR. ROBERTS: Dr. Hayes, I know you are going to move into  
11 another aspect of your presentation.

12 In terms of planning for a break, long do you think your next  
13 presentation, which I guess is the last phase of your presentation,  
14 formal presentation will take roughly speaking?

15 DR. HAYES: Depends on how many times we stop for  
16 questions. Maybe an hour. I have no concept --

17 DR. ROBERTS: In that case, let me suggest that we take a very  
18 short break, about 10-minute break. And then move into the last  
19 phase of the presentation. Let's reconvene in 10 minutes.

20 (Thereupon, a brief recess was taken.)

21 DR. ROBERTS: Before we continue with Dr. Hayes'

1 presentation, I would like to take just a moment for some public  
2 recognition of somebody who has worked very hard for the last seven  
3 years behind the scenes to make meetings like this possible.

4 Shirley Pursuval (ph) has worked on the SAP staff and has been  
5 frankly somebody who has really helped us get these meetings  
6 together, has made sure all of our travel gets done, we get reimbursed  
7 for travel, which I can say is very important to us on the panel, and  
8 those of us on the permanent panel wanted to take just a moment to  
9 recognize her service.

10 She is retiring. This will be her last SAP meeting. On behalf  
11 of the panel, we wanted to thank Shirley very much for everything she  
12 has done for us over the years.

13 (A flower arrangement was presented.)

14 MS. SHIRLEY: Thank you very much. It has been a pleasure  
15 working with you all.

16 DR. ROBERTS: Dr. Hayes, before you start, let me just give a  
17 heads up, an announcement.

18 We have a number of other individuals that wanted to comment  
19 make public comments. I wanted to alert the other public  
20 commenters that it is my intention to take most, if not all, of the  
21 public comments today so that the panel can begin our deliberations

216

1 tomorrow morning.

2 I would like to request that you be prepared to stay as long as it  
3 takes for us to get through the public comments today.

4 DR. HAYES: I'll be quick.

5 DR. ROBERTS: That was not intended as a message to you, Dr.  
6 Hayes.

7 I just wanted to give everybody a heads up that we do want to  
8 try and get through the public comments today because we have a lot  
9 of things to talk about. We want to be able to start on those tomorrow  
10 morning.

11 With that, Dr. Hayes, please continue.

12 DR. HAYES: So the real question is I guess what does it mean,  
13 and we have talked about what is robust, what is not robust both in  
14 terms of robust data and whether or not populations are such.

15 In a recent exchange, the following statement was made, the  
16 basic tenets required -- in terms of what does it mean, the first thing  
17 we have to do, really establish, is there cause and effect to get  
18 through the data and decide do we have enough data to determine  
19 whether or not there is cause and effect.

20 This is -- I'm now reading from a quote, I believe it is Solomon  
21 and Carr, the basic tenets required for establishing causal

1 relationships between environmental factors and disease were  
2 formalized nearly 40 years ago. And I underlined the word required.

3 And I have another quote here, what I do not believe -- this is  
4 from someone else, is that we can usefully lay down some hard and  
5 fast rules of evidence that must be obeyed before we accept cause and  
6 effect.

7 None of my nine viewpoints can bring indisputable evidence for  
8 or against the cause and effect hypothesis, and none can be required  
9 as a sine qua non. That's actually Sir Bradford Hill himself saying  
10 this so-called Hill criteria, the nine Hill criteria that they can't be  
11 required.

12 I do realize, though, that some of the nine criteria are  
13 important. And yesterday, Dr. Vandercrack (ph) talked about and  
14 spoke about Glen Fox and the use of the Hill criteria. I've also  
15 spoken to Glen Fox.

16 What I'm going to do now is I'm going to put this in that kind of  
17 framework. I'm going to use the so-called Hill criteria. Dr.  
18 Vandercrack didn't go into detail. But there are sort of nine criteria.  
19 What I'm going to do is I'm going to talk briefly about each one and  
20 talk about whether or not we have met any of these criteria and the  
21 value of them.

1 Strength of association, and I'll go through and tell you what  
2 each one is, consistency, specificity, temporality, biological gradient,  
3 plausibility, coherence, experimentation and analogy.

4 These are all from Hill's 1965 address.

5 They are all gray now. I'm going to go through each one,  
6 explain what is required in order to meet that criteria.

7 And then when I think it is met, I'm going to make it black and  
8 bold. And then we'll move on to the next one.

9 First, is there a strong association. Like I said, I hope that I  
10 have explained well enough and now made clear how we have decided  
11 what is a gonadal abnormality.

12 In most cases, they are -- even if they are below 10 percent, in  
13 most cases they are morphologies and the histologies,  
14 histopathologies that we never see in controls with the exception of  
15 the three animals in one tank out of 300 where we found the  
16 unpigmented ovary and controls.

17 In addition, if I'm correct, and we'll go through this a little bit  
18 later, if I'm correct that these are males, that it is only the males that  
19 are being transformed in this way, then the actual percentage of males  
20 is doubled.

21 So if it is 10 percent of a population and it is only males, then

1 it's actually 20 percent of the males that are being deformed this way.  
2 And I do believe that that is the case. So we're probably  
3 underestimating the number.

4 Dr. Kelley and I had a discussion. We have in place an  
5 all-genetic male producing line that we're doing that kind of work  
6 with.

7 I think the association is made strong by the number of  
8 replicates, by our protocol for doing things double blind. Even if we  
9 only mix three pairs of animals, each time we replicate our  
10 experiments multiple times, especially now when I know at some  
11 point I will have to come here and address and defend papers the way  
12 most people --

13 The other thing is we have, when I say tens of thousands, this is  
14 a couple shelves double stacked deep of slides. They are all available  
15 to anybody who wants to see any of those slides.

16 So the sample sizes aren't small. Even when we're talking  
17 about five percent of animals had this morphology, three percent had  
18 this, we have huge sample sizes, both from our field collected data as  
19 well as in our laboratory associated data.

20 And I think it at least in terms of the stuff that we're doing that  
21 there is a strong association. I know in the field there are some

1 problems with dose effects and what we find doing. And we're doing  
2 more to extend that data set.

3 One of the big things that has come up at this meeting that I  
4 want to address is sort of consistency. There are seventeen studies. I  
5 haven't seen them all. And certainly there are some published studies  
6 and past studies.

7 I want to spend a little bit of time addressing the consistency. I  
8 think that's at the heart of this meeting.

9 Whether or not there have been consistent effects, I have to be  
10 frank. I was a little surprised when I read the white paper that said  
11 that weight of the evidence didn't support and that there were no other  
12 studies supporting the gonadal problems.

13 And in part, for example -- I mean another scientist wrote to  
14 me, Tyrone, I agree with you that the important issue is for everyone  
15 involved to come to grips with and stop minimizing the fact that  
16 independent laboratories have demonstrated an effect of atrazine on  
17 gonadal differentiation in frogs. There is no denying this.

18 That was an e-mail from Jim Carr who is the lead scientist on  
19 the Ecorisk panel. That really wasn't the -- in terms of consistency,  
20 that wasn't the message that I have gotten today.

21 What I'm going to do now is I'm going to go through those

1 studies, some of those studies being Carr studies, and talk about why  
2 there might appear to be inconsistencies and how we might evaluate  
3 all of those studies together, at least in my opinion.

4 The study that we all just got done talking about is the PNAS  
5 study where in that paper I identified a percentage of hermaphrodites.  
6 We did not try to classify them in any way. Now we have done that.  
7 That's available for anybody who wants to see it.

8 Speaking of which all of my data -- John Ashby said it was  
9 impossible to reproduce. All of my SOPs have been available to the  
10 Ecorisk panel. They were developed with the Ecorisk panel, with  
11 Novartis, Syngenta. The whole three-day renewal was developed with  
12 them. All of my data has been available. I have mailed all of my data  
13 to them multiple times. I have mailed them thousands of slides more  
14 than once.

15 So all that has been available. And I'm available if they wanted  
16 to evaluate that to try to solve those problems.

17 That brings me to another study, something else that was said  
18 here today that wasn't true. These are just a scan of some data sheets.  
19 It's just an image here.

20 If you look there, you will see names TBH, GMM, Gloria  
21 Maglena Mendoza (ph) slide was here today. These are data sheets

1 from work that I conducted with Ecorisk and Syngenta in 1999.

2 If you look down the sex column in those data, you will see  
3 question marks and i's indicating gonadal abnormalities. Although,  
4 again, we hadn't classified them and found them then, we started  
5 producing these kind of data in exactly the same design as early as  
6 1999.

7 So those are two studies now, both done by me, one with  
8 Syngenta, Ecorisk funding. One without.

9 There is also the work by Tavera-Mendoza. The effect is  
10 different. But still, it shows an impairment of gonadal development  
11 with exposure to atrazine. The exposure was different. It was done  
12 under different conditions, which I will address. But it is still  
13 consistent with atrazine having a negative impact on gonadal  
14 development in xenopus laevis. There are three studies now looking  
15 at xenopus laevis.

16 The Carr, et al., study published in Environment Toxicology  
17 and Chemistry is going to take us a little bit of time.

18 In part, despite that in February the statement, there is no  
19 denying this, was made some of the problems in comparing the Carr  
20 and Hayes study are demonstrated here. Carr says in April 22, wrote  
21 to me, you are right. We have not repeated your work, which was

1 admitted by Dr. Ashby today, and I remind everyone that I talk to  
2 about this, and did mention this clearly at the EPA briefing that I  
3 attended in March.

4 The problem is there were a lot of differences between the  
5 studies. And these differences, some of them explain why they got  
6 what appear to be different effects. And some of them I think are  
7 quite detrimental to their study, but I'll have more to say about that  
8 later.

9 At one point on April 22nd, though, he also said after saying he  
10 didn't -- oops, we're back there again. Sorry about that.

11 What I want to do is now point out what those differences really  
12 are and what they really mean. So this is the same figure I showed  
13 you earlier with low testes hermaphrodites and unpigmented ovaries  
14 shown on it.

15 And these are the data that I've pulled out of Carr's study. I  
16 stacked them as a bar. It's a little bit different. The lobe testes are  
17 what he called discontinuous testes.

18 And probably the hermaphrodites from the pictures I have seen  
19 from his work, the hermaphrodites that are included there probably  
20 include unpigmented ovaries if you look at the photograph in the  
21 paper as well as some of what we would call hermaphrodites.

1           One of the points I want to make is the total percent of gonadal  
2 abnormalities at 25 parts per billion is not all that different than what  
3 we find at 25 parts per billion.

4           A couple things have to be addressed, however. Now you are  
5 looking at against just the Carr data. Not only do we see that there  
6 are effects at the 1, 10 and 25, but it almost looks like there is a dose  
7 response.

8           But there is a problem with the dosing. I think when we go  
9 through the problems with the dosing, you are going to see why some  
10 of the discrepancy arose.

11           I raise my animals in four liters of water, 30 animals in four  
12 liters of water from the time that they hatch until they metamorphose.

13           In the Carr study, somehow 60 animals were maintained in 100  
14 mils of water initially. What I'm going to do now is do a little  
15 comparison at the so-called 25 parts per billion or 25 micrograms per  
16 liter dose.

17           What that means for me is that in four liters I have 100  
18 micrograms of atrazine. So the concentration is 25 micrograms per  
19 liter. 100 micrograms and four liters.

20           What that means for Carr is that he is actually adding two and a  
21 half micrograms of atrazine to his animals. Meaning that I'm adding

1 40 times more atrazine at my 25 parts per billion dose.

2 When you consider that Carr has twice as many animals, my  
3 animals are actually getting 80 times as much atrazine. It is the  
4 difference between -- I have two kids. You saw that already. It is the  
5 difference between giving a spoonful of cough syrup to one of my  
6 kids or dividing it between the two.

7 The concentration is the same, but the dose is different. This is  
8 especially important when we consider what I just told you, that  
9 USGS and I have shown that every three days the amount of atrazine  
10 is decreasing by 30 percent.

11 That's probably a factor of the number of animals that the  
12 atrazine -- that are being exposed.

13 By the time we get out to -- I forget how many days, I think I'll  
14 show that in a minute, he has eight times less atrazine. And by the  
15 time we come to the end of this study, he has only half as much  
16 atrazine being added to his animal in terms of micrograms of atrazine  
17 per tadpole.

18 The concentration is the same, but the dose is different. And it  
19 is actually worse because he only changes half the water every three  
20 days. So he is actually only adding half as much as he says he is  
21 adding.

1           If we look at the critical stages when the gonads are  
2           differentiating, that means that Carr's dose is actually 16 times lower  
3           than mine. So even though we both looking at 25 micrograms per  
4           liter, he has a fourth of volume of water, twice as many animals and  
5           only he only adds half the water -- or changes half the water each  
6           time.

7           And certainly with the atrazine decreasing by 30 percent every  
8           three days, then this is significant. So his 25 -- my 25 parts per billion  
9           dose is more like a one and a half parts per billion dose, again, even  
10          though the concentration is the same.

11          This becomes important when we start to look at steroid  
12          hormones as well. This is data that I published back in 1995 showing  
13          that over 24 hour periods tadpoles, for example, in the case of  
14          steroids, metabolized the steroids very quickly, and two tadpoles can  
15          metabolize eight time faster than one tadpole, such that a couple  
16          micrograms of estradiol would disappear in a matter of a few hours,  
17          for example, when added the *xenopus laevis* tadpoles, which is  
18          probably why the estradiol didn't work in those treatments that were  
19          conducted by Carr.

20          So the first step is to correct the doses. If we correct the doses  
21          now, then this sort of low dose effect problem starts to go away. His

1 1.5 should be compared to my one part per billion dose. He has a .6  
2 part part per billion dose which is more better compared to my .8.  
3 And then he sort of falls below .1, which is where we start to see the  
4 effect.

5 Then Carr did something called Cochran Armitage Test, which  
6 my understanding is is looking for a linear dose response.

7 For discontinuous testes, he got a P value .0003. For  
8 hermaphrodites, he got a P value of.0042.

9 And most biologists, I think, would accept .05. But Carr  
10 referred to P values as low as this to weak trends, Carr, et al., in the  
11 paper published in SETAC.

12 I asked Carr how can a P value of.0003 be a weak trend. His  
13 response was, well, it's a weak trend because when the data from the  
14 top dose are dropped, the effect is no longer significant.

15 So what I'm understanding now is that we're looking for a linear  
16 dose response in an experiment that has three doses. We're  
17 eliminating the top dose, the one that is really in the range that we're  
18 trying to look for. And now we have two data points which makes it  
19 even difficult to draw a line.

20 So the statement that they haven't repeated the effects or found  
21 significant effects I believe is a little bit faulty given the procedures

1 that have been used to be able to say that.

2 Furthermore, if you look through that SETAC paper, there are a  
3 number of things that are significant or that are considered significant  
4 at P less than .05.

5 These are quotes, those animals reaching NF stage 66 first in  
6 each tank were significantly larger. Estradiol treated animals, but not  
7 males were significantly longer than ethanol treated males, P less  
8 than .05. The percentage of intersex gonads also was significantly  
9 greater in the estradiol treated group, P less than .05.

10 So clearly other points in the SETAC paper we accept. Carr, et  
11 al., accepts P less than .05. But whenever the effects were associated  
12 with atrazine, they were referred to as weak trends.

13 For example, they showed increased edema with atrazine  
14 exposure, with a P value of .02, abnormal swimming with a P value of  
15 .0004, inhibition for foreleg emergence.

16 So there is the other reference to inhibition of metamorphosis  
17 in xenopus laevis with a P value .03, inhibition of tail reabsorption by  
18 atrazine with a P value of .04, increased discontinuous gonads, as I  
19 said, with a P value of .0003, increased intersex gonads with a P value  
20 of .0042. And originally, when the paper was submitted there was an  
21 increased laryngeal muscle size with a P value of .033, but that was

1 different when the paper actually came out.

2       There are some other issues, and right now I'm still talking  
3 about the Carr paper, but I'm moving towards the white paper because  
4 I have some concerns about this bioloading thing and the flow through  
5 thing.

6       And I'm going to use now some information from the Carr paper  
7 because I think we're making an error.

8       If we look at the first half of my animals to metamorphose, they  
9 can be any experiment, I don't remember where I pulled these data  
10 from, and the first half in the Carr experiment, they are about the  
11 same size in terms of body weight. They are approaching .6 grams.

12       If you look at the last half of the animals -- I just looked at the  
13 first half literally and the last half to metamorphose, they are slightly  
14 larger.

15       And we just had this discussion. If you take longer to  
16 metamorphose, you should be larger. Whereas if you look at the last  
17 half in the Carr study, they tend to be smaller.

18       So we have a huge host of adverse effects, many of them  
19 associated with atrazine, that I didn't see, the edema, abnormal  
20 swimming, inhibition to metamorphosis, as well as a growth curve  
21 that I just talked to you about as being something that I only see when

230

1 the animals are exposed to multiple pesticides or multiple stressors.

2 In this case, we're actually looking at controls.

3 Here is a bigger problem. Again, here is the first half of the  
4 animals to metamorphose in the Carr study in body weight. There is  
5 the last half.

6 And now what I'm plotting up for you is one you were asking --  
7 somebody was asking me about the size of my animals. There is 3000  
8 data points from one big study we did this year.

9 Again, it shows the longer the animals take to metamorphose,  
10 the larger they are. There is regression through all points. Some of  
11 these are treatments. These aren't just controls. Some of these are  
12 treatments, controls, males, females. I put everybody in there just so  
13 I can show you what the sizes should look like.

14 There is the average in the Carr, et al., study. Out of 3000  
15 animals, I have a single animal that approaches the average size that  
16 Carr reported in his study.

17 And I think this is the condition that led the EPA in the white  
18 paper to decide that there were poor conditions and bioloading  
19 problems.

20 In addition to that, only 30 percent of the animals  
21 metamorphosed in 80 days. Essentially, in excess of 50 percent of the

231

1 animals were terminated. And we don't accept anything below 85  
2 percent survivorship. Not mortality. And on average, we get 90  
3 percent.

4 What the white paper presented was something that looks like  
5 this. Now, this is a different kind of figure. This is developmental  
6 stage looking at wet weight. This is metamorphic climax.

7 And this is really the data set we're interested in. This is the  
8 animals at stage 66.

9 I guess the recommendation was either four animals per tank --  
10 but if we're trying to look at sex ratios, we cannot set up a protocol  
11 that requires to have four animals per four liters, or whatever it was.

12 I also think, as other people have expressed, that raising  
13 animals on a flow through is unnecessary, which I'm going to show  
14 some data suggests.

15 I think it will generate a lot of problems, with all due respect.  
16 xenopus don't like it. It will be an expensive set-up. It will mean  
17 that anybody who wanted to do EPA acceptable research cannot do so  
18 without industry funding or somebody who is going to pay the big  
19 bucks.

20 It would generate a huge water loss. It would generate a huge  
21 amount of waste, depending on what chemical you are trying to

1 dispose of and dealing with, a huge amount of waste. A huge cost to  
2 pay for the chemical to go through flow through.

3 It would eliminate the ability to move between comparative  
4 studies. Some animals simply won't live on flow through at all. And  
5 it would also -- we would be starting over from ground one.

6 We're talking about differences in conditions now. If we start  
7 over next year with a required flow-through system, everything we  
8 have done now will be done under different conditions and you will be  
9 throwing it out.

10 Everything that Vitche and everybody else did it will be  
11 difficult to compare based on what I'm seeing here at the meeting  
12 now.

13 In addition, it is, again, with all due respect, it is not required.

14 The recommended time for healthy animals in the white paper is  
15 55 days, metamorphic size at .63 grams. That's when you're looking  
16 at animals at stage 66.

17 So what they are recommending is that on flow through you can  
18 get animals at stage 66 that metamorphose by 55 days that come out  
19 on average at .63 grams.

20 There is 3000 data points, all raised in static renewal, every  
21 three days, the average is .67 grams in size and they metamorphose

1 average in 46.3 days.

2 So talk about weight of the evidence. There is 3000 data  
3 points.

4 The problem is not the bioloading and static renewal in the  
5 Carr, et al. and some of the other studies.

6 You're looking at a tank after three days getting ready to be  
7 renewed. That's what a tank looks like if the animals are fed properly  
8 after three days. I want you to notice two things.

9 One, I want you to notice how dirty the water is and imagine  
10 only changing half that water for 100 days. The other thing I want  
11 you to notice is all this stuff from someone in Michigan state. I've  
12 seen a picture of the set-up. There are no lids on the tanks. That's  
13 probably the source of the contamination in controls.

14 All this stuff that is on the back of our lids here. If there are no  
15 lids, it would be in the tank that it's sitting next to. Stuff grows even  
16 when you are scrubbing them and cleaning them every three days,  
17 which what we do.

18 Now you are looking at the color-coded net, you are looking at  
19 the plastic we lay down to prevent contamination, color-coded lid,  
20 color-coded tank. Those are scrubbed every three days. You have to  
21 take all the water out to scrub them.

1           The other problem that Carr, et al., experienced was the  
2 feeding. Again, you are looking at our study design on the bottom, 30  
3 tadpoles in four liters.

4           Carr apparently fed his animals .4 grams every three days, if  
5 I'm getting that right from the paper.

6           We start off at .32. So his animals are unfed by 1.6 -- oops, 4.8  
7 because they are only fed every three days. And then we increase the  
8 amount of food as the animals grow.

9           So that by the end, by days 21 to 80, our animals are getting 48  
10 times more food than the animals in the Carr study were, which is  
11 probably why they weren't growing and the high mortality.

12           In addition, what disturbs me the most is -- what you are  
13 looking at now, this is a study that's looking at food levels. This was  
14 our standard food level. That's what the one X 1 was. We tried half  
15 that, 2.55 times -- you're looking at survivorship.

16           On this side you are looking at growth for these different food  
17 levels. That's our food level that we use in the blue. That's the food  
18 level used by Carr, Novartis, Syngenta, Ecorisk in the yellow.

19           Essentially, this was their food level. There is the high  
20 mortality. What disturbs me is this study was done for Syngenta.  
21 They all had all these data available. We turned in a final report. So

1 this feeding regime was developed as a part of the Ecorisk panel when  
2 I was a part of it. So these data were available.

3 I don't know why the animals were underfed by a factor of 48 or  
4 why they weren't changed. My point is please don't use that as a  
5 model for how we're going to conduct studies in the future, because  
6 the flow through is going to generate a lot more problems and it's  
7 really going to set us back, it's really going to set us back to starting  
8 all over again.

9 Anyway, inspite of all that, as I pointed out earlier, the gonadal  
10 abnormalities are still there. There was some statistical manipulation.  
11 But I think that this study is consistent with the other studies that  
12 have shown that even under those conditions -- I agree the conditions  
13 were poor, but I don't think you throw the study out. Even under  
14 those conditions, some of the same gonadal abnormalities were  
15 identified.

16 Again, here is testes and ovaries, multiple testes and ovaries  
17 identified with strong strength of association, P value .0003.

18 Coady, et al., was a study at Michigan state -- another thing  
19 that frustrated me yesterday was a long discussion about ethanol  
20 effects and effects in controls and oocytes in controls.

21 These are some data that I obtained from Michigan state on

1 nominal atrazine doses. There is 25 parts per billion. The 25 parts  
2 per billion nominal atrazine dose is in excess of what it is supposed to  
3 be with large pair.

4 What I'm mainly concerned about is that if we blow this up,  
5 there is controls, there is .1 part per billion. There is as much  
6 atrazine in the controls as there is -- in fact, there is four times .1 part  
7 per billion atrazine in the controls.

8 So when we're having these discussions about background  
9 hermaphroditism and how many oocytes are in the controls and is  
10 there an ethanol effect, I think data like these need to be upfront so  
11 we will know what some of the confounding effects were.

12 Nevertheless, I don't think -- again, I think there are problems  
13 with the study. There were no controls. But the effects that they are  
14 finding are consistent with the effects that other and better controlled  
15 studies also found.

16 There is the work that we did in nature. I know that we'll talk  
17 about some of the dose effects, field effects, things like that. This  
18 was the same work that we published in EHP. It was the longer  
19 version of the paper. The nature paper was published as a shorter  
20 version. We were allowed to publish more of the gonadal  
21 abnormalities so that you can actually see the full range of what we

1 looked at there.

2 There is the Reeder, et al., study. This is actually how I first  
3 learned about testicular oocytes. Here is an animal with testicular  
4 oocytes. Here is an animal with testes and ovary. This study showed  
5 gonadal abnormalities similar to what we're looking at in cricket frogs  
6 associated with atrazine exposure.

7 It has been dismissed because it had a P value I think of .06 or  
8 .07 or something like that.

9 But I think -- by the way, Hill didn't like statistics, if you go  
10 back and read. He didn't think we needed to rely on them.

11 But if you look at the whole body of evidence that's building, I  
12 think we can probably accept, especially if we're going to error on the  
13 side of caution, I think we can accept the .067 P value, which is what  
14 I think he got for the association.

15 Finally, there is a study of McKoy, et al., on the toads. One  
16 thing I want to point out -- this came out I think after the SETAC  
17 meeting in Utah. It was said about this study that lends credence to  
18 University of Berkeley endocrinologist Tyrone Hayes' hypothesis that  
19 atrazine is affecting sexual development of amphibians.

20 Gross, that would be Tim Gross, Dr. Gross of the Ecorisk panel,  
21 added that their findings are consistent with the previous work of both

1 Hayes and Texas Tech experimental toxicologist James Carr, Carr  
2 finds an effect at atrazine concentrations that are similar to what we  
3 see in the field and to what we think the toads are exposed.

4 I really didn't get that feeling here today that we were all -- that  
5 Texas Tech and Jim Carr and Tim Gross and everybody was in  
6 agreement that there were gonadal effects.

7 I guess the other thing I want to point out, and then I'm going to  
8 move on is that not only do we have nine studies show associations,  
9 but 1, 2, 3, 4 -- I don't know about the seventeen that came in now,  
10 four of those studies are Novartis, Syngenta, Ecorisk studies.

11 And at least two of the people involved in those studies, two of  
12 the lead authors twice have said that we were all in agreement. There  
13 is no denying this, James Carr said.

14 The other thing I want to point out is -- I'm not familiar with  
15 weight of evidence. But my feeling is no study is going to be perfect.

16 In every study, you should have measured this thing, you should  
17 have done that or you should have designed it this way.

18 We have nine studies, all imperfect, but we have nine studies  
19 all supporting the same endpoint, that atrazine has an effect on the  
20 gonads.

21 Sure. We can find a problem with my study. I can probably find

1 a problem with the Carr study. We can find a problem with the Gross  
2 study. But they all point towards the same thing.

3 I find it hard to believe that it is going to be a quintessence --  
4 actually, I was expecting more.

5 Let me skip to the important part. At any rate, relative to other  
6 things that are going on with respect to new data sets that you have  
7 not seen yet, this is Jim Carr to me, I think that the past arguments  
8 over larynx, et cetera, will become trivial. There will soon be bigger  
9 issues to address. And I think that the biologists will end up on the  
10 same side on these issues. Believe me on this. Any biologist will not  
11 be able to ignore the data that will soon be coming out. This is from  
12 the leadoff in the panel.

13 My concern -- and I'm not accusing anybody, but my concern  
14 actually is in the other paragraph, is that, we either haven't seen  
15 anything or because there is a line here, there is a lot going on that  
16 you don't know about. Trust me on this. My differences with other  
17 panel members have to do with how the new data are interpreted. I am  
18 a biologist. Others will be using statistics to minimize the impact by  
19 the new data sets.

20 So my concern is that either we haven't seen everything or  
21 everything hasn't been presented. Because I thought from this I was

1 really expecting to see something much more robust. And now the  
2 presentation seemed to be that there is nothing robust.

3 At any rate, back to my other point, Hill himself said, I would  
4 myself put a good deal of weight upon similar results reached in quite  
5 different ways.

6 So all of this discussion about the weaknesses in the studies  
7 being that they were all done under different conditions and bla, bla,  
8 bla, Hill himself saw as a strength. If all of these studies, each with  
9 their own independent flaw, are all pointing to the same thing, doesn't  
10 that add something to the weight of the evidence?

11 Furthermore, Fox, who Dr. Vandercrack cited, said, in  
12 ecoepidemiology, the occurrence of an association in more than one  
13 species and species population is very strong evidence for causation.

14 Here, over the studies we have just looked at, we have  
15 multiple studies in the pipidae. We have several studies in ranidae.  
16 We have at least one bufonidae study and then we have Reeder's stuff  
17 on hyalodae.

18 It's not only that we're seeing effects under different condition,  
19 under different experimental regimes, but these effects are spread out  
20 across anurans and different families even.

21 I don't know how we can ignore the strength of that data as

241

1 described by Hill himself, as described by Fox himself as sort of  
2 mandated by the panel that that's how we review the data.

3 The specificity I'm going to kind of skip over, because even  
4 Hill so much didn't hang on this. Specificity sort of required that  
5 there be one cause, one effect.

6 And we all know that one chemical doesn't do one thing. I'll  
7 come back to the end of that.

8 I guess the only thing I want to say relative to that is all of the  
9 effects are on the gonad. They might be manifested in different ways  
10 because the gonads develop differently in the different species, but  
11 there are specific effects that seem to target males, that seem to  
12 involve demasculinization and feminization and, again, achieved in  
13 different ways.

14 The temporality criterion requires that the cause come before  
15 the effect.

16 There are several things we need to address here. In a  
17 laboratory experiment, it is really sort of a moot point. I guess it is  
18 not a moot point. The point is we don't see the abnormalities until the  
19 animals are exposed to atrazine. So the cause in this case because  
20 we're delivering it does come before the effect.

21 Other problems come in the field. There is two types of

1 temporality issues. That is one is if you are looking at animals in the  
2 field, were they exposed to atrazine when they were developing. I  
3 have already shown this. I will go through it quickly. I just want to  
4 blow up one section. We have already talked about that.

5 If you look at a map like this, a diagram like this, the eggs are  
6 being laid in March to April at the particular site that I chose. And  
7 then as somebody pointed out, around my birthday in late July, that's  
8 when we're going back to collect the metamorphs. At the time we left  
9 (ph) eggs at this particular site -- atrazine level's above .1 level. And  
10 at the time we went back to pick them up, they were up at 15.

11 So it is not likely that the atrazine disappeared. Especially,  
12 given that the larvae were growing up during this peak. It is not  
13 likely that the atrazine disappeared during the critical stages that  
14 we're concerned about.

15 So here I don't think there is a temporality issue. And I have  
16 already shown you that when you go back here, even before any  
17 atrazine is applied, the atrazine from last year is still measurable  
18 above .1 micrograms per liter. So the animals are likely exposed  
19 during this period.

20 The other temporality issue is did this these abnormalities, if  
21 they are abnormalities, occur before the advent and use of atrazine.

1           So for example, I'm studying these abnormalities here in 2001,  
2           '2, '3.

3           Reeder, et al., has gone back -- I don't think it has been  
4           published yet, but has gone back through museum specimens and  
5           shown that he gets some of these gonadal abnormalities dating all the  
6           way back to 1940.

7           atrazine, of course, doesn't show up until 1960. But other  
8           estrogenic compounds did show up about 1940, which in this new  
9           paper by Val Beasley (ph) he discusses the coincidence of an increase  
10          in gonadal abnormalities associated with DDT exposure, and then  
11          there is the potential of another increase associated with atrazine,  
12          with atrazine use.

13          One thing that comes up a lot that I disagree with is Vitchie  
14          even earlier than this talked about a I guess what he called a sex  
15          changing frog that started out hermaphroditic. It's a European ranid.  
16          Many of the papers were written on the same population. It wasn't  
17          multiple populations.

18          The other thing is this morphology is not one that we have been  
19          looking at or one that has been described at all. And it may very well  
20          be a natural occurring phenomena, but I don't believe it is the same  
21          phenomena that we have been looking at.

1           In addition, I want to point out that Hill himself said it does not  
2 -- I'm sorry. This is Rothman and Greenland, 1998, it does not follow  
3 that a reverse time order is evidence against the hypothesis that C can  
4 cause D. Rather observations in which C followed D merely show  
5 that C could not have caused D in these instances; they provide no  
6 evidence for or against the hypothesis that C can cause D in those  
7 instances in which it precedes D.

8           So in other words, even if you go back and find, oh, yes, there  
9 were some hermaphrodites before the use, it still doesn't rule out that  
10 atrazine is the cause now, that atrazine isn't increasing the incidence.

11           Here is the one that everyone wants to talk about, it seems,  
12 biological gradient.

13           Biological gradients suggests that there should be some kind of  
14 dose response, some kind of concentration or dose relationship  
15 between the cause and effect.

16           What I have heard and what I was disappointed to read,  
17 actually, in the white paper, what I disagreed with is that it is almost  
18 made a requirement if atrazine really does this thing that there has to  
19 be as is typical in toxicology a monotonic linear dose response.

20           If you make that a requirement, as an endocrinologist, I know  
21 can tell you you will never, I would guess, never nail any endocrine

1 disrupter and never get to the mechanism. I'll show you why.

2 Fox, again, who Dr. Vandercrack cited, says, causal  
3 relationships need not be linear or monotonic. That's just what I said,  
4 actually.

5 Hill in 1965 even acknowledged this and said we should then  
6 need to envisage some much more complex relationship to satisfy the  
7 cause and effect hypothesis.

8 I think we need to take this advice that Hill gave us in 1965.

9 Fox went on to say, in sum, there is a marked threshold.

10 And I hope I'm going to convince you that that's what we're  
11 looking at now and why that's why we're looking at.

12 Others are Sigmoid. He said, yes, yet others are parabolic.

13 I think we need to really take this to heart. Because I think that  
14 hormones don't work this way. I think I can provide you with enough  
15 examples.

16 Let's look again at the larynx data, data that we looked at  
17 earlier. Where there appears to be a threshold effect, whether you are  
18 looking at absolute laryngeal size at one part per billion, the slide  
19 said 10, it should have said 1, at 1 part per billion, you get threshold  
20 effect where the larynx is smaller than controls, but then it doesn't get  
21 smaller as you go out, I assume is the concern of everyone.

1           And even if you look at the percent above the mean, you get the  
2           same effect. At one part per billion, it levels out. Even though this is  
3           going from 80 to 90 percent, it is essentially, I would argue, a  
4           threshold effect.

5           Here is I think why. Here is what happens in a control female.  
6           You should recognize that. That's a gonad. That's a larynx. And in a  
7           control female, there is no testosterone. The larynx doesn't show this  
8           accelerated growth.

9           In a control male, testosterone stimulates the larynx to grow as  
10          shown in this schematic.

11          Now let's look at atrazine treated male at low doses and high  
12          doses. In an atrazine treated male, testosterone, potential, we'll talk  
13          about other mechanisms, potentially is converted to estradiol,  
14          reverses the gonad, and then there is no testosterone to make the  
15          larynx grow.

16          In an atrazine treated male with a high dose of testosterone,  
17          testosterone is converted to estrogen and the animal's is reversed. So  
18          the larynx doesn't grow.

19          It is not that atrazine is shrinking the larynx. It is preventing it  
20          from growing. Once you prevent it from growing, you can't prevent it  
21          from growing more. It is done.

1 Giving a higher dose of atrazine can't take away more of  
2 something that has already been taken away. I'll show you in a minute  
3 it has been taken away. We have a lot more data on hormone stuff  
4 that I think Dr. Kelley will be more pleased with as well.

5 So I don't know how you could expect with a higher dose, if we  
6 accept that this is a plausible mechanism, that the larynx will get  
7 smaller and smaller.

8 You get some dose. In this case I will argue it's one part per  
9 billion. You get some dose that diminishes testosterone. The larynx  
10 doesn't grow. Giving a bigger dose won't make it not grow more. It is  
11 already done.

12 Let's address this other problem now. Again, just bear with me.  
13 I'm not going to argue that it is a parabolic or an inverted U, whatever  
14 you want to call it, but we clearly did see bigger effects at the low  
15 dose than at the high dose when we looked at gonadal abnormalities in  
16 *rana pipiens*.

17 Again, let's sort of stick with the testosterone estradiol  
18 hypothesis again. And now we're going to talk about mammals for a  
19 second. I'm going to give you an analogy.

20 Normally, gonadotropin releasing hormone from the  
21 hypothalamus stimulates the gonadotropin's FSH and LH from the

1 pituitary. Now we're talking about a female. Those hormones  
2 regulate the ovarian cycle.

3 Estrogens in the ovarian cycle are necessary for regulation of  
4 the uterine cycle and estrogens are necessary for follicle growth and  
5 development. In other words, you need estrogen for follicle growth  
6 and development.

7 In fact, if you look at ovulation, estrogens increase, increase,  
8 increase. You hit some threshold effect and you ovulate.

9 Giving more estrogen early, giving a bigger dose of estrogen  
10 won't make you ovulate more. It won't make you ovulate faster.

11 In fact, if you give a big dose of estrogen, you will set the  
12 whole thing down and not ovulate at all.

13 You would never see a monotonic dose response in what  
14 everybody in this room understands. Throughout your menstrual  
15 cycle, estrogen levels increase, increase. They hit a peak. They hit a  
16 threshold, and you ovulate.

17 That threshold is different from woman to woman. It might be  
18 different from month to month, but it is not a dose response. You  
19 give a big dose of estrogen, try and make that happen earlier, you  
20 will shut the whole thing down. That's how birth control pills work.

21 We can envision, again, it's my hypothesis now, that we are

1 working on, that GNRH stimulates gonadotropins in frogs. Those  
2 gonadotropins normally in a male would stimulate testosterone.  
3 atrazine turns on aromatase. And then estrogen results in  
4 development of oocytes. Results in oogenesis.

5 On the other hand, if you give a bigger dose of atrazine, it is  
6 very well possible that you make enough estrogen that you shut down  
7 the pituitary and you get no oocytes.

8 So at this low dose, you would support oogenesis. At the high  
9 dose, you would shut it down or inhibit it potentially.

10 I think we have to really start thinking about what we know  
11 about endocrinology and integrating it, marrying it with toxicology in  
12 a way it will allow us to not slap on a requirement of a monotonic  
13 linear dose in order to generate cause and effect.

14 The other thing that is going to happen is if you look in the  
15 field -- we talked about several problems, other chemicals in the  
16 field, the levels of fluctuating up and down in the field both  
17 temporarily spatially.

18 Now if we accept that there are threshold effects, now we  
19 accept that there potentially could be parabolic effects -- and again,  
20 we are working on it right now, multiple populations in our lab using  
21 multiple doses of both estrogen and atrazine, but you can imagine that

1 this threshold effect, the percent you see of hermaphrodites might be  
2 here, for example, in terms of the dose response at one site. You  
3 might have the same threshold for another population, but a greater  
4 proportion of animals respond. You might have overlapping limits.  
5 You might have some limits that don't overlap.

6 So there is -- it would, if this is true, make it almost impossible  
7 with different -- sorry, varying sensitivities between populations,  
8 varying patterns of gonadal development, which we have already  
9 documented across populations, varying degrees of resistance and  
10 hybridization across populations, fluctuating atrazine levels.

11 I don't think you can ever expect to see if we go to a site with  
12 high atrazine and we find high hermaphrodites. I think we would be  
13 very misguided if that's what we were looking for.

14 But I think we need to look for the association between atrazine  
15 contamination and what we can deem as gonadal abnormalities. Get  
16 those animals from those populations back in the lab and ask did they  
17 develop that way naturally or is there something identifiable in the  
18 field and something that we can do in controlled laboratory studies.

19 Again, at least one member of the panel agreed. Here talking  
20 about mechanism, Jim Carr says, without this information we will not  
21 be able to determine why not all animals respond the same way, why

1 threshold responses differ, and if testicular oocytes observed in frogs  
2 inhabiting ag areas are due to atrazine.

3 So at least two of us are starting to think that way. I really  
4 want to encourage the panel -- because I honestly don't think we'll  
5 find the kinds of monotonic linear dose responses that we have been  
6 talking about here.

7 And Hill, of course, acknowledged, often the difficulty is to  
8 secure some satisfactory quantitative measure of the environment  
9 which will permit us to explore this dose response.

10 So it is going to go be difficult. Again, we have just gone  
11 through all of this. Even if you are taking sites one day to the next,  
12 you can get huge differences in atrazine levels.

13 If we are going to try to build a dose response curve, which one  
14 of these concentrations would we use to build that curve on.

15 Again, I think in a data set like this I agree. It is all over the  
16 place. All we can say is where there is atrazine there is gonadal  
17 abnormalities. Pull these populations into the laboratory and find out  
18 what we can learn there.

19 So I think that if we're looking for a monotonic linear response,  
20 we won't be able to make this black and bold. But I think if we use  
21 the endocrine system as a model and look carefully at the

1 mechanisms, then I think we can do this as well.

2 The other big question. Plausibility and coherence. Is there a  
3 plausible mechanism that's coherent with the types of effects that  
4 we're describing.

5 We have proposed one. And again, we proposed that because of  
6 what is known in mammalian cell lines, et cetera.

7 That atrazine turns on aromatase, that converts testosterone to  
8 estradiol and results in demasculinizing effects because of the loss of  
9 androgen and feminizing effects because of the gain of estrogen.  
10 Where is the evidence?

11 So again, here is our proposed mechanism you have seen before,  
12 that the inappropriate expression of estrogen causing effect on the  
13 gonads and the lack of androgen causing effect on the larynx.

14 Let's talk about the gonads first. One of the reasons that we  
15 believe that this is a plausible mechanism is that we have a pretty  
16 extensive data set on estrogen treatment, not just our data, but a  
17 historical data set. Controls, in controlled males, we would expect no  
18 ovaries and a normal or male type larynx.

19 In estrogen treated animals, we would expect them all to be  
20 female and maintain a normal female larynx.

21 We did a study -- actually, Roger Lou (ph) did this study

1 starting back when he was in undergraduate, a former student in the  
2 lab. Dr. Kelley and Brackenridge were talking actually about this  
3 exact kind of study. You are looking at animals in days.

4 We did a study where we treated animals with estrogen from  
5 stage 50 to stage 66. Or we treated for one week from stage 50 to  
6 stage 53 and then kept them without treatment. Or we treated them  
7 for two weeks, stage 55 to 66 was without treatment.

8 So everywhere you see green the animals were treated with  
9 estradiol. So they were treated for a week after hatching and then  
10 allowed to grow up, treated for two weeks after hatching then allowed  
11 to grow up or treated throughout the larval period.

12 This shows the different treatments from one week, two week  
13 and the full larval period.

14 You are now going to look at the sex ratio. This is phenotypic  
15 sex based on gonads. You are looking at the number of males and  
16 females.

17 Here is a control. You are going to look -- males are in blue.  
18 Females are in green.

19 I'm going to show you a line for 50 percent. Controls are  
20 roughly 50/50. That looks like about 40 percent female, 60 percent  
21 male.

1           Now I'm going to show you different treatments that we talked  
2 about. When we treat animals from stage 50 to stage 66, we get 100  
3 percent females.

4           When we treat animals now from stage 50 to 55, this is that  
5 two-week treatment, we get females, we get a few males and then we  
6 get some of these abnormalities. I'll show you what that is.

7           And then when we treat for this one-week period, we get  
8 predominantly females, about 70 percent, some males and then we get  
9 this group of abnormalities, about 20 percent.

10           So this incomplete dosing in terms of the duration of dosing  
11 gives us abnormalities. Those abnormalities look like this.  
12 Discontinuous gonads or lobed testes and animals that have both  
13 testes and ovaries of varying types. Some varying types of  
14 hermaphroditism.

15           Somebody asked about the unpigmented ovary, however. What  
16 I'm telling you is that we can induce lobed testes and hermaphrodites  
17 with estrogen.

18           So those, again, I think support the hypothesis that the animals  
19 are being inappropriately exposed to estrogen. The morphologies are  
20 identical to what we see with atrazine when you give them incomplete  
21 exposure.

1           There is another treatment that we can give, and we actually get  
2           33 percent unpigmented ovaries, other than atrazine and the three  
3           control animals out of 300, sporadin (ph) acetate, the anti-androgen.  
4           That's the only compound I have ever treated with when I find these  
5           unpigmented ovaries.

6           So what I'm telling you is a normal male presumably has some  
7           testosterone coming from his testes and he is all good. What you are  
8           looking at now, this is the unpigmented ovary. This is the mixed  
9           hermaphrodite with testes and ovaries. And this is the so-called  
10          broken testes or lobed testes or discontinuous testes.

11          What I'm suggesting is that atrazine, by depleting testosterone,  
12          results in the unpigmented ovary. I'm not saying that atrazine acts  
13          like sporadin acetate.

14          I'm saying a compound that blocks androgen action produces  
15          the same effect as atrazine which takes away -- which decreases  
16          androgen. And that estrogen exposure induces these other types of  
17          abnormalities that we have identified.

18          What is the evidence for effects on steroidogenesis? We did a  
19          one time measure with four animals in each treatment group. That is  
20          the data that were presented in the PNAS that we talked about today.  
21          But we have done a number of other things, a number of other studies

1 to address the effects of atrazine on steroidogenesis in adult males.

2 You will notice the fonts, et cetera, have changed. This is a  
3 presentation that was given by Melissa Lee that I'm using to present  
4 the data she has been working on for the last year.

5 The first thing she addressed was how to optimize conditions to  
6 best measure plasma testosterone concentrations. In this case, instead  
7 of decapitating animals, we used cardiac puncture.

8 We evaluated a number of things. I was unhappy, I have to say,  
9 with the field studies that were presented by Dupree (ph), by the  
10 Ecorisk panel.

11 Trapping the animals in traps and holding them for unspecified  
12 amount of times can affect hormone levels. We, for example, went  
13 through a lot of, as you can see here, through a lot of work to make  
14 sure that there was no association between our handling time and  
15 effects on hormone levels. There wasn't. So we addressed that.

16 We addressed housing effects. We asked whether or not  
17 animals should be housed singly or in groups. We did that over a  
18 number of days. The green are the group housed, and the blue are the  
19 single housed. They don't care whether they have roommates or not.

20 We looked at daily fluctuations to figure out what time of the  
21 day. So the gray shows nighttime measurements.

1           And these are just controls. These are actual, I forget, four or  
2           five animals -- Melissa? Is that about the sample size? Four or five  
3           animals, different groups of animals bled at each time. And then we  
4           bled some continuously shown in yellow to look at the effect of  
5           handling.

6           As I was telling Dr. Kelley, these are nighttime levels, are  
7           much higher than what we measured in the PNAS paper.

8           How do we characterize the effect of atrazine on hormonal  
9           profiles over time. That was just how we evaluated how to do the  
10          studies. Here is a number of other studies we did. We exposed  
11          animals to atrazine. Up to 72 days. We took blood samples at all the  
12          data shown here.

13          Here are some of the other differences I told you about. This is  
14          a Berkeley colony. This is a colony that we have maintained for more  
15          than 10 years. You're going to look at Berkeley control males. They  
16          do fluctuate. When we put them into the experiment, they almost  
17          always initially go down and then come back up and start cycling.

18          When you expose animals to atrazine, and these are all 10 parts  
19          per billion exposures, I believe, when you expose animals to atrazine,  
20          they decrease and they never go back up again.

21          Part of the point I want to make is that if you measure at the

1 wrong time during the experiment, like here for example, you will get  
2 no effect. But you are getting no effect -- in this case, you would be  
3 getting no effect because the animals aren't in season at this time,  
4 whatever that means, whereas clearly here there are significant  
5 effects.

6 Here is another example. Nasco animals. If you look at those  
7 levels, those are Berkeley animals. They also tend to have smaller  
8 larynges than the Nasco animals. They are not as masculine or  
9 something as the animals we order from Nasco. But even at their  
10 peak, they are at about seven nanograms per mil.

11 If we order animals from Nasco and acclimate them and look at  
12 control males, this is interesting, first of all they crash. They come to  
13 Berkeley. We so-call acclimate them and they crash.

14 If you look at the atrazine treated animals, they also crash. But  
15 this study was done before we knew to look at night. If you look at  
16 nighttime samples over the same thing, they are incredibly different  
17 at the nighttime.

18 Again, if you look at the wrong time during the wrong part of  
19 the year or during the wrong part of the day, you won't see the effects.  
20 But non effects aren't because the atrazine treated males are doing  
21 okay. It is because you are looking at the wrong time for the control

1 males.

2 We started, we haven't finished yet, but we started addressing  
3 another question that has come up. I think the Dr. Kloas brought it up.  
4 Maybe it is not this aromatase. Maybe it's interference with  
5 steroidogenesis some other way.

6 So at what point in a hormonal axis is atrazine acting. This is  
7 Melissa's diagram of the hypothalamus showing pituitary stimulating  
8 the gonad to make testosterone. And we're suggesting that that  
9 testosterone is being aromatized. But it is also possible that  
10 testosterone isn't being made, that the gonadotropin -- Dr. Cooper has  
11 certainly shown some effects on higher up in the axis.

12 So we did a little study. We treated animals for 27 days. What  
13 I'm going to show you is -- we took blood samples at these time  
14 points. And here is again day on the X axis. Plasma testosterone on  
15 the Y axis. Control females. We did measure females. We can't  
16 detect that atrazine does anything with females. They hardly have any  
17 testosterone.

18 And here now are the control and atrazine-treated males.  
19 Controls are in black. atrazine's in red.

20 So again, during part of the cycle, you can detect differences.  
21 During part, you can't. Then right here we took half the animals from

1 each group and we injected them with HCG. So we inject them with  
2 gonadotropin.

3 We asked, even those these atrazine treated males have  
4 diminished testosterone relative to these control males, can they  
5 respond to a pituitary challenge. We inject with HCG. There is the  
6 control males. And there is the atrazine treated males.

7 So the testes can respond. In fact, Melissa and I have argued  
8 over this, but it looks like they are responding in the same way in  
9 terms of the magnitude of the response before and after with controls  
10 as well as with the atrazine treated ones.

11 Here is the individual data that I promised to show you for the  
12 aromatase stuff. When we are getting activity, it is highly variable.  
13 We have now moved to an invitro system where we can better control  
14 things -- I mean, a total invitro exposure system, the whole thing.

15 We get highly variable from individual to individual,  
16 experiment to experiment. All I can tell you is we don't tend to see  
17 high or significant aromatase in controls. And we are still chasing  
18 that part of the mechanism.

19 We are also entertaining the possibility that there are other  
20 mechanisms acting and perhaps even more than one mechanism.

21 So the important things to take out of this are that you really

1 have to know the cycles and watch where you sample. You can cycle  
2 on one day and get an effect and on another day not get the effect.  
3 But if you don't get the effect -- by the way, this big arrow bar is due  
4 to one animal that had a huge amount of testosterone.

5 If you don't get the effect, it is not because the atrazine animals  
6 are recovering. It is because you are sampling at the wrong time, at  
7 least in our experience.

8 Also, you have to keep track of the fact that these are nocturnal  
9 animals. Day sampling, as we did for most of our stuff, is probably  
10 not the most appropriate time table to sample the animals.

11 Also, there may be other mechanisms working, because HCG  
12 can at least stimulate probably to the same extent an atrazine exposed  
13 male as a control. Again, there might be multiple mechanisms.

14 Experimentation, what Hill suggested here was that sometimes  
15 you can do an experiment. And he was mainly talking about  
16 epidemiology. If people are getting sick because they go to the well,  
17 shut the well down and see if the illness goes away.

18 We have talked quit a bit about experimentation. I think we  
19 have quite a bit of evidence. The only other experiment I guess would  
20 be to take atrazine away and then do field work and see if the  
21 hermaphrodites go away. That might be something to do through

1 temporality.

2 The last one I want to address is analogy. What does analogy  
3 really mean. What does analogy tell us about cause and effect.

4 Carr and Solomon in the learned discourses exchange also said  
5 that it was unlikely that atrazine caused these problems because, I  
6 quote, atrazine is a potent phytotoxic compound specifically designed  
7 to target a mechanism of action unique to plants, the binding of  
8 plastoquinone II during photosynthesis. As such, there is no a priori  
9 reason to suspect that atrazine would affect endocrine function in  
10 vertebrates.

11 So the idea seems to be that the pesticide is specific, so why  
12 would you expect it to have these kinds of effects that we're talking  
13 about.

14 As an analogy, I'll use DDT. It was pretty specific in what it  
15 did to insects. It inhibits mitochondrial ATP synthase, but DDT and  
16 its metabolites also inhibit prostaglandin synthesis, bind the estrogen  
17 receptor as an agonist, bind the androgen receptor as an antagonist,  
18 bind sex hormone binding globulin, induce aromatase, increase  
19 progesterone synthesis, inhibit glucocorticoid synthesis.

20 So here is another compound by analogy that has a pretty  
21 specific mechanism, but it does a lot of other things as well. If

1 atrazine does more than one thing, I don't think it should preclude us  
2 from exploring what it does to vertebrate -- in vertebrate sex  
3 differentiation.

4 The other analogy is there is huge literature, I think mostly out  
5 of Japan showing that there is a number of triazines pharmaceutically  
6 used that specifically inhibit aromatase. So by analogy, we have  
7 triazine.

8 I'm going to speak very briefly and then I'm done.

9 By analogy, we have triazine such as atrazine that we know at  
10 least in mammalian systems induce aromatase. We know in some rat  
11 model systems it will induce estrogen dependent or tumors associated  
12 with estrogen, estrogen exposure.

13 And by analogy, we have aromatase inhibitors that are being  
14 designed and tested in estrogen dependent cancer cell lines that are  
15 being designed specifically to do just the opposite.

16 On the one hand, we have tri-ines that we believe turn on  
17 aromatase and are associated with things like gonadal abnormalities  
18 and mammary cancer. And then we have triazines that we know inhibit  
19 aromatase but do just the opposite.

20 I think that analogy should help guide us as well in  
21 understanding it and understanding the mechanism.

1           Again, I want to point out Fox's point, in ecoepidemiology, the  
2           occurrence of an association in more than one species and species  
3           population is very strong evidence of causation.

4           I have pointed this out already. We have evidence, field,  
5           laboratory in the pipidae, the ranidae, the bufonidae, the hyaloidea  
6           done under all kinds of different conditions, all kinds of different  
7           exposures.

8           And every one -- we can argue about a .067 statistic if we want.  
9           We can argue about a lack of monotonic dose response. We can argue  
10          about all the flaws for all of those individual studies.

11          But when you line them all up together, every one with its  
12          flaws, whether the animals are healthy or not, they are still producing  
13          gonadal abnormalities with significant P values.

14          What about the mechanism? This has come up, the work of  
15          Sanderson, et al. But there has been a pretty detailed proposed  
16          mechanism. Again, GnRH stimulates the pituitary to release  
17          gonadotropins. The gonadotropins stimulate steroidogenesis through  
18          a G protein that turns on adenylate cyclase, results in a production of  
19          cyclic AMP, and, through a number of steps, turns on Cyp 19, which  
20          is the gene for aromatase, and aromatase, of course, converts  
21          testosterone to estradiol.

1           This whole thing is controlled over this side by  
2 phosphodiesterase. This is all proposed by John Giesy and  
3 Sanderson, et al.

4           And phosphodiesterase gets rid of the cyclic AMP, converting it  
5 to AMP. That's what keeps this whole system from going crazy.

6           Giesy and Sanderson, et al., propose that atrazine ties up the  
7 phosphodiesterase somehow. And the result is an elevation of cyclic  
8 AMP, an elevation of aromatase and increased estrogen.

9           So this has all been done, proposed and work done in cell lines.  
10 It has been shown in rats that estrogen increases in rats that are  
11 exposed to atrazine.

12           It has been also shown in rats that pituitary is down-regulated  
13 by that increase in estrogen. This is work done primarily by  
14 Syngenta. And it has also been shown that estrogen stimulates the  
15 release of prolactin.

16           So atrazine-fed rats tend to have high prolactin, high estrogen  
17 and low pituitary gonadotropins.

18           Is this mechanism that we know in some detail in mammals  
19 relevant to any of the studies that we are talking about now, relative  
20 to this issue?

21           In part, I promised you I would come back to it, in part, the

1 connection was made by Dr. Vandercrack. If we know something  
2 about the mammalian androgen receptor, it should tell us something  
3 about frogs.

4 Now we're going to make that point again. If we know  
5 something about the mechanism in mammals, does it tell us something  
6 about frogs.

7 Elevated estrogen -- we have already made the argument, i'm  
8 not going to hit you over the head again, is associated with the  
9 hermaphroditism, at least 11 studies. Again, I didn't read the  
10 seventeen that just came in.

11 The decrease in testosterone associated which, I think we have  
12 shown -- we have good evidence for, is associated with the laryngeal  
13 growth. These are both my studies. Industry funded and not industry  
14 funded.

15 The prolactin and estradiol both -- estradiol has a strong  
16 inhibitory effect on metamorphosis. And prolactin inhibits  
17 metamorphosis.

18 So if a mechanism like this is working in amphibians, it might  
19 explain effects in inhibited metamorphosis. I'm sorry. I forgot to put  
20 the reference in, but it is in the paper that I made available to the  
21 SAP.

1           It has been shown in *Ambystoma tigrinum* that there is an  
2 inhibition of metamorphosis with a P value less than .05.

3           And *Xenopus laevis*, Carr, et al., showed inhibition of foreleg  
4 emergence, P value of .03, inhibition of tail reabsorption, P value .04.

5           In *Rana clamitans*, McKoy, et al., interestingly enough showed  
6 an inhibition of metamorphosis at 25 parts per billion, acceleration at  
7 10 parts per billion.

8           And Kelly Haston in my laboratory in work that's ongoing now  
9 has shown inhibition of metamorphosis in some populations in *Rana*  
10 *pipiens* and not others, which we have already talked about.

11           This mechanism and these changes in hormone levels that we  
12 know in some detail in mammals would explain many of the effects in  
13 amphibians.

14           In Sanderson, et al., John Giesy as coauthor wrote, a logical  
15 concern -- this is based on the mammalian work. A logical concern  
16 would be that exposure of wildlife and humans to atrazine herbicides,  
17 which are produced and used in large quantities, and are ubiquitous  
18 environmental contaminants, may similarly contribute to estrogen  
19 mediated toxicities and inappropriate sexual differentiation.

20           So this has been proposed by a member of the panel previously,  
21 the observed induction of aromatase, the rate limiting enzyme in the

1 conversion of androgens to estrogens, may be an underlying  
2 explanation for some of the tumor promoting properties of these  
3 herbicides in vivo.

4 So now we're going back again. I'm telling you just as we  
5 know things in mammals that help us out in frogs, now knowing  
6 something in frogs may be telling us something more about problems  
7 with environmental health, but more importantly, public health.

8 Namely, there was a study in 1990 that showed exposure to  
9 atrazine resulted in significantly increased incidence of mammary  
10 tumors, which respond to both estrogen and prolactin, and  
11 inflammation, sometimes with abscess formation of the prostate  
12 gland, which also responds to prolactin and estrogen.

13 And elevated prolactin and estrogen has been shown in rats.

14 In another rat, the sprague dawley female, Charles Eldridge  
15 wrote in 1999, nine years after the Pinter study in 1990 -- so in 1990  
16 Pinter showed increased mammary tumors and inflammation of the  
17 prostate glands in male Fischer rats in 1990.

18 In 1999, Eldridge reported mammary tumors in sprague dawley  
19 rats, and wrote, the mammary tumor response is limited to one strain  
20 of one species in females.

21 Then again, Stevens, et al., in 1999 wrote, the carcinogenic

1 effect of high doses of atrazine observed in the female sprague dawley  
2 rat is a strain, sex and tissue specific response that does not have  
3 biological relevance to humans.

4 So my only point is we now have prostate and mammary cancer  
5 that we have known about in rats since 1990. We know that these rats  
6 have elevated estrogen and prolactin and decreased pituitary  
7 hormones.

8 We now have effects in amphibians that we're looking at, what,  
9 13 years later that tend to be associated with the same hormones.

10 So this mechanism, again, my point being may be telling us  
11 about a lot more than frogs. I believe this meeting is about a lot more  
12 than frogs.

13 There is studies coming out. I have seen the abstract on  
14 induction of brain aromatase in fish in response to atrazine. Tim  
15 Gross of course with the panel in 1999, 2000 showed elevated  
16 estradiol, decreased androgens and vitollegenin in male exposed fish.

17 This effect is consistent with the effects in amphibians and with  
18 the hormone measurements. It is consistent with the some of the data  
19 that has been discussed, the reptile data. It is consistent with the  
20 effects in mammals, the effects in rats, elevated estrogen and  
21 prolactin and decreased androgen associated with these types of

1 cancers.

2 With regards to the relevance in humans, in one study atrazine  
3 exposure decreased intrauterine growth in Iowa communities with  
4 contaminated water. P value less than .001.

5 And this is just a few. atrazine exposure increases testicular  
6 cancer and prostate cancer in hispanic, R equals .41, and black, .67  
7 farm workers, Mills 1998. Effects associated with, again, estrogen  
8 and prolactin with regards to the prostate.

9 atrazine exposure in drinking water increases breast cancer  
10 with a P value of less than .0001. Again, a disease associated with  
11 elevated estrogen and prolactin.

12 Coming up on the last side, atrazine exposure increased  
13 prostate cancer 9.4 times in a Novartis plant in Louisiana, again, an  
14 effect that has been associated with increased aromatase and  
15 prolactin.

16 So we have more than effects in just amphibians consistent with  
17 the proposed mechanism, fish, four major classes of amphibians and,  
18 again, data that I will suggest in a place like this in Africa where the  
19 runoff is the water that they use for cooking, I think if you told the  
20 people in that village that their water was causing some of the kinds  
21 of effects that we're debating here, then I think there would be cause

271

1 for concern because they know that water comes to their home. The  
2 same is true for us.

3 With regards to amphibian sensitivity, I think our canary is  
4 trying to sing. And we should listen.

5 DR. ROBERTS: Thank you, Dr. Hayes. Let me now open the  
6 presentation to questions by the panel.

7 Dr. Green.

8 DR. GREEN: Regarding the feeding adjustments that you made  
9 that you feel are critically important in some of your studies, did you  
10 base that on trial and error in your lab or published anuran kilocaloric  
11 requirements?

12 DR. HAYES: When I became involved with Syngenta -- the  
13 way we used to operate was we changed the water and renewed the  
14 solution every day, every 24 hours. We would come in at 4 a.m.  
15 change all the water.

16 When I initially -- I can't tell you why, but when I initially  
17 started operating with Allen Hosmeran (ph) with the panel, they didn't  
18 want such frequent renewal. You would have to address Syngenta  
19 Ecorisk to find out why.

20 So we did an initial study where we tried to do the change every  
21 three days, and we found high mortality, low growth and all the

1 animals died.

2 We filed a final report in 1998. We terminated the study  
3 because we created an 85 percent mortality. That was our first  
4 atrazine study that we conducted. The final report was filed with  
5 Syngenta.

6 Then we did a study called 98XL Food. We did two studies.  
7 98XL Food 1 in 2. The final reports were filed for those studies.

8 Those studies looked at the food level that we used to feed, that  
9 was the 1 X, that we used to feed when we did the changes every day.  
10 Then we went half that because we were worried about water quality  
11 and twice that and four times.

12 So that it was based on what we did, but with a different  
13 frequency of water change. All of that was made available. The  
14 studies were signed off on and finalized by Syngenta and Ecorisk.

15 DR. GREEN: What is the food?

16 DR. HAYES: We feed Purina rabbit chow to everything. With  
17 the xenopus, we grind it up and dissolve it in the water. With other  
18 animals we feed it as whole pellets, and it's weighed out whether it's  
19 ground up for xenopus or whether it's thrown directly in a tank. It's a  
20 weighed set amount per number of tadpoles, as you saw.

21 DR. ROBERTS: Other questions?

1 Dr. Kelley.

2 DR. KELLEY: In reading over the Carr, et al., study, it is true  
3 that in their methods they say that they fed them, whatever it was, .4  
4 grams a day. But in a later part of the paper, in the results paper they  
5 said that they actually checked the amount of food that was there.  
6 And if it was clear, they fed them more food.

7 So they do look like they were underfed because they were at 22  
8 degrees and they took so long to go through metamorphosis.

9 But I just wanted to correct that impression. The paper does  
10 look like they did adjust the food for the animals during the course of  
11 the study.

12 DR. HAYES: That may have been my oversight.

13 DR. ROBERTS: Dr. Isom, then Dr. Matsumura, then Dr.  
14 Richards.

15 DR. ISOM: Perhaps I missed this, but did you measure estrogen  
16 levels in that study?

17 DR. HAYES: Yes. We have tried to measure estrogen levels.  
18 We have measured -- Melissa, correct me if I'm wrong, we can  
19 measure estrogen levels in females. We have never measured  
20 circulating estrogen in adult males.

21 DR. ISOM: You could not detect it--

1 DR. HAYES: We have never detected it. We could not detect  
2 it. That's right. It was below the detection limit.

3 Correct, Melissa?

4 And we have also tried measuring whole body and larvae. We  
5 have been unable to measure estradiol.

6 DR. ISOM: Why do you think you can't detect in the males, the  
7 ones that were treated? To me, it's logical if you are converting  
8 testosterone to estrogens and you are seeing the high levels at night,  
9 you would see the end product of that.

10 DR. HAYES: Not necessarily.

11 Again, estrogen has some role normally in the testes for sperm  
12 development, but you don't find it circulating in males.

13 There is local production of estrogen where it has its effect --

14 DR. ISOM: I would have to ask our endocrine people that.  
15 What is the half life? How long would the estrogen stay around? You  
16 see the fluctuation the diurnal or the changes in testosterone. But  
17 does estrogen stay around longer? And would it build over time or  
18 you would see higher levels of that?

19 I think that's important for the hypothesis to consider that.

20 DR. HAYES: But I don't think that elevated circulated estrogen  
21 is necessarily a requirement. Again, we're also looking at other

1 mechanisms that may be acting.

2 An increase in estrogen is consistent with the effects on the  
3 gonad, it's consistent with the oogenesis and what appear to be  
4 vitolleginic oocytes in rana pipiens. But we haven't -- all I can say is  
5 we have tried many times. We haven't been able to detect it.

6 DR. ISOM: What would you recommend, then, for an  
7 experimental design that we should consider to do a real complete  
8 endocrine work-up on these animals to validate this hypothesis,  
9 support this?

10 DR. HAYES: In our place right now, we're focusing on  
11 measuring up-regulation of Cyp 19 and doing the aromatase assays  
12 invitro.

13 Because it is very possible, just like in the brain of males, it is  
14 very possible that the aromatase and the conversion occurs locally  
15 and never goes into circulation.

16 So we're focusing right now on tissue expression of the gene for  
17 aromatase and biochemical activity.

18 DR. ISOM: What I tissue would you recommend to look at?

19 DR. HAYES: We have been doing it in the gonads. But since  
20 reading some of the stuff and John Giesy and others are doing, the  
21 brain is actually a better choice of tissue. We haven't started working

1 with the brain yet.

2 DR. ISOM: Thank you.

3 DR. ROBERTS: Dr. Matsumura.

4 DR. MATSUMURA: Have you checked specific inhibitors for  
5 those systems that you are proposing like aromatase inhibitors or PKA  
6 inhibitors? Have you done that?

7 DR. HAYES: Right now, we have just initiated a study that  
8 looks at atrazine in combination with miconazole, which is an  
9 aromatase inhibitor that we know to work in other frogs in our  
10 laboratory. So we are doing something like that now.

11 I guess your proposal is if you give atrazine plus an aromatase  
12 inhibitor, would you prevent the effect. I have two students right now  
13 who are working on that problem.

14 DR. MATSUMURA: I also noticed that when you added the  
15 HCG, atrazine treated ones did not recover as much as the control did.

16 DR. HAYES: I have argued with my colleague about this. I say  
17 the -- I guess one point is if you look at the percent response from  
18 where they started, it is the same. But the atrazine treated ones don't  
19 go up to where the controls are.

20 DR. MATSUMURA: What do you think?

21 DR. HAYES: We have only used -- that study is only done with

1 one dose of atrazine, and we've only done one dose of HCG.

2 Maybe if we give a bigger dose, maybe it will be a dose  
3 response. Maybe if we give a larger dose of atrazine, they will be  
4 able to recover. What it tells us is that the animals are still able to  
5 make testosterone. Suggests that the problem might be at the  
6 pituitary.

7 Did we try to measure estrogen after the HCG injection,  
8 Melissa?

9 I would imagine we would have. So the testes isn't ruined. It's  
10 able to respond. And the response is -- in terms of where it started  
11 and where it ended up is the same, but it doesn't go up to match the  
12 control.

13 DR. ROBERTS: Dr. Richards, then Dr. Coats, Dr. LeBlanc,  
14 then Dr. Denver.

15 DR. RICHARDS: I bring this up just because you used the data  
16 source a couple times. Most recently with respect to your arguments  
17 about temporality. It's the USGS data on atrazine concentrations.

18 Those data represent concentrations in the main stem  
19 Mississippi and its major tributaries.

20 Probably have little or nothing to do with the kinds of  
21 concentrations, exposures you would see in farm ponds or ditches in

1 the upper midwest.

2 The Mississippi, of course, is responding to hundreds or  
3 thousands and thousands of square kilometer watersheds.

4 The little ditches are responding to a square kilometer or less.  
5 I would expect the concentrations or the exposures would be much  
6 more episodic as you have raised in some other issues.

7 But it's just a poor data set, I think to try to characterize the  
8 temporality.

9 DR. HAYES: Initially, the way we used that was just like the  
10 atrazine used based on sales. We wanted a basis before we go out and  
11 rent two SUVs and an 18 wheeler and spend a whole month away. Are  
12 there levels that have been measured. Are there sites. And do we  
13 have reason to believe that the levels might be highest during those  
14 times.

15 Certainly, in the runoff, right off the cornfields where we're  
16 collecting and right off the rivers, I think that the timing is at least  
17 relevant, if not the levels, that they are applying in March, late  
18 March. They are going to be highest.

19 DR. ROBERTS: Did you have a follow up, Dr. Richards?

20 Dr. Coats.

21 DR. HAYES: Sorry. Remember, we're also taking

1 measurements before and after at the site. We're not counting on that  
2 as our measurement.

3 DR. COATS: Just wanted to follow up on the doses again and  
4 the measured concentrations. You didn't say measured concentrations  
5 in the PNAS paper.

6 And it would be very helpful if those were published, I would  
7 think. Did you have -- and you said you had decay over a three-day  
8 period.

9 DR. HAYES: Sorry. That wasn't from the PNAS paper. Those  
10 are brand new data that I just did with Battaglin maybe within the last  
11 six or seven months.

12 DR. COATS: Are those data available?

13 DR. HAYES: I can make those data available. Those are brand  
14 new data. Those weren't done along with the PNAS paper.

15 The question had come up -- put some discussions between  
16 myself and members of the Ecorisk panel about static renewal and bla,  
17 bla, bla. So we did a study where we maintained the animals or  
18 maintained buckets without tadpoles and took the measurements.

19 That's fairly recent data.

20 DR. COATS: Without tadpoles, you took the measurements?

21 DR. HAYES: With and without tadpoles. It is associated with

280

1 the tadpoles.

2 DR. COATS: And how much decay did you get over a three-day  
3 period?

4 DR. HAYES: It goes down by 30 percent.

5 DR. COATS: Was that in the .1 part per billion or 25 part per  
6 billion?

7 DR. HAYES: We did two doses, as I recall. It was a 25 and a  
8 .1.

9 DR. COATS: And it was the same percentage decay over both  
10 times?

11 DR. HAYES: Yes.

12 DR. COATS: My other question was about the summer set that  
13 you looked at, metolachlor and atrazine together. Was that at 25 parts  
14 per billion?

15 DR. HAYES: No. We looked at atrazine and --

16 I don't remember the proportions off the top of my head, but  
17 they were mixed at exactly the same proportions. They were mixed in  
18 bicep.

19 We've also conducted the studies where they were mixed at the  
20 proportion that we find them in the field, which is close to what you  
21 find in --

281

1 DR. COATS: At what concentration?

2 DR. HAYES: We looked at one at .1, and we've looked at one  
3 at 10, I believe, in initial study. And in a study we just completed, we  
4 did .11 and 10 in a xenopus laevis study.

5 DR. COATS: The graph you showed us about the maturation  
6 rate, body weights, which concentration was that at or was that a  
7 pooling of all?

8 DR. HAYES: Which?

9 DR. COATS: You showed the summer chemicals, atrazine  
10 metolachlor. You showed a --

11 DR. HAYES: That's the .1. Anything higher than that died.  
12 You're talking about --

13 DR. COATS: Not of the 10 mixture. Just of the atrazine and  
14 metolachlor.

15 DR. HAYES: That's .1.

16 DR. COATS: Thank you.

17 DR. ROBERTS: Dr. Hayes, as a short follow up to one of Dr.  
18 Coats' questions for clarification.

19 To what extent are your data available to EPA as they try and  
20 sort through this? If they pick up the phone and say, hey, can we look  
21 at your data on -- to what extent --

1 DR. HAYES: Depending on what you want, I have already sent  
2 a huge amount of data and SOPs and protocols through Tom Steeger.  
3 But anybody in this room is welcome to get raw data, transcribed  
4 data. Every animal I have talked about is available in the lab. Every  
5 slide is available in the lab. You can anything you want. It's a public  
6 university. It is all yours. Anything you want, just call and let me  
7 know.

8 DR. ROBERTS: Thank you.

9 Dr. LeBlanc and then Dr. Denver and Dr. Green.

10 DR. LEBLANC: I would like to revisit the issue of the  
11 hypothesis that atrazine increases aromatase activity and thus the  
12 conversion of testosterone to estradiol.

13 I agree with you that an increase in serum estradiol really isn't  
14 a requirement of the hypothesis that the induction could result in  
15 tissue specific induction and tissue specific increases in estradiol  
16 levels.

17 But with atrazine treatment, you observed a tenfold decrease in  
18 serum testosterone. So the question is where is the estradiol.

19 DR. HAYES: I would love to show a beautiful graph with lots  
20 of estradiol.

21 DR. LEBLANC: It should be up in serum. And it is not.

1 DR. HAYES: Like I said, we're exploring others mechanisms.  
2 The most consistent with the effects that we see, the feminizing  
3 effects and the demasculizing effects is that hypothesis, which is  
4 supported in other vertebrates. That's why we focused on it and we  
5 have focused on --

6 DR. LEBLANC: Perhaps what you should consider, I think you  
7 are really, is that you might be getting tissue specific increases in  
8 estradiol levels, but, in addition, you might be seeing some separate  
9 decrease in testosterone synthesis.

10 And I think your data with respect to evening testosterone  
11 levels supports that. In control animals, you see an increase in  
12 testosterone levels, which is in all probability due to an increase in  
13 synthesis.

14 With atrazine treatment, that doesn't occur, implying synthesis  
15 isn't occurring. Increased synthesis isn't occurring. It has been cut  
16 off in some manner.

17 DR. HAYES: We have worked with Doug Stocko (ph, the  
18 person who is doing molecular biology in my lab, has a whole host of  
19 cyp genes, not just cyp 19, but also steroidogenic acute regulatory --  
20 the star proteins he's looking at and a number of other genes or a  
21 number of other enzymes are also being examined.

1 DR. LEBLANC: It may tie in with the LH hypothesis as well,  
2 that it may be that you are, as related to testosterone synthesis, you  
3 are interfering with the LH surge that might be controlling  
4 testosterone synthesis.

5 DR. HAYES: Certainly, the HCG injections, they're  
6 experiments that we need to repeat, but the HCG injections support  
7 that.

8 DR. ROBERTS: Dr. Denver.

9 DR. DENVER: I have two questions. I agree that the endocrine  
10 literature supports high doses of hormones actually having either a  
11 lower effect, no effect or even sometimes an opposite effect. We  
12 found that in my laboratory with certain assays.

13 Regardless of whether the effect of atrazine is endocrine  
14 mediated or not, your data in rana pipiens shows that the higher dose  
15 actually results in a lower incidence of gonadal abnormalities.

16 So would that lead you to predict, then, that, if you went to the  
17 field, that in sites with higher atrazine contamination you would have  
18 lower incidences?

19 DR. HAYES: I think it would depend on the population and the  
20 response of that population. As you know, rana pipiens, low doses of  
21 estradiol do nothing. A slightly higher dose of estradiol make 100

285

1 percent females. Really high dose of estradiol I think at the  
2 milligram levels make 100 percent males.

3 So given that the natural steroid can have that kind of  
4 variability in its effects, I don't know how it will make that  
5 prediction.

6 I think that's actually a strength of what we have done in the  
7 field paper, not a weakness, and said, look, you can't expect to find  
8 these kinds of relationships. Or maybe it means that there is not a  
9 correlation.

10 DR. DENVER: My second question has to do with the field  
11 studies. I appreciate the difficulties of interpreting field data.

12 And I was wondering if you've considered the possibility that  
13 there may be the other estrogenic compounds that are not necessarily  
14 pesticides, for example, PCBs that may be responsible for the  
15 variability that you see across different sites.

16 DR. HAYES: Absolutely.

17 DR. DENVER: Have you addressed the contamination from  
18 PCBs, for example, in these different sites? Or are any data --

19 DR. HAYES: We've had PCBs and organochlorines measured in  
20 the other studies, but not in the current studies, we have not.

21 We focused on what we knew was being applied there now to

1 try and generate -- there were other things like nitrate. For example,  
2 atrazine interacts with nitrate, and nitrate interacts with other  
3 chemicals.

4 Just already the size of the study was so enormous that we can't  
5 put everything in.

6 DR. DENVER: So there is a possibility that at least the  
7 incidence of intersex or whatever you want to call it at the different  
8 sites may be due to entirely different phenomena.

9 DR. HAYES: It could be -- phytoestrogens could be involved  
10 for that matter. It could be a host of a number of things.

11 We're limited also in what we can have analyzed chemically  
12 because of the cost. We have only analyzed for compounds that the  
13 farmers report that they used at the property.

14 We didn't go through and do a sweep of analyze for 100 things.

15 DR. DENVER: Well, as you got closer to industrial areas, did  
16 you see any -- I didn't recall the incidence. I'm wondering if you saw  
17 a higher incidence closer to industrial areas as you moved east  
18 perhaps?

19 DR. HAYES: No. I don't recall there was any relationship that  
20 way either.

21 DR. ROBERTS: Dr. Green.

1 DR. GREEN: Could you elaborate on the concerns you have  
2 about potential future studies that propose using flow through tanks  
3 and specifics about your concerns, the rate of water turnover, for  
4 example, and the detrimental effects it might have on tadpole  
5 development.

6 And then if we propose future grow-out experiments, do you  
7 think that flow through tanks might be acceptable for juveniles and  
8 adults?

9 The reason I ask is because these are space consuming and  
10 labor intensive experiments. As you know, the flow through systems,  
11 while expensive, give better control over water quality and are less  
12 labor intensive and they work quite well for adults.

13 But I need to hear your opinion specifically about what is  
14 wrong with the flow through tank for developmental stages.

15 DR. HAYES: I guess one of the differences -- I'm a basic  
16 scientist at a public institution who primarily count on things like  
17 National Science Foundation, which don't fund applied studies, that  
18 count -- funding is pretty difficult.

19 When I think about the cost of -- some of these compounds are  
20 expensive. The cost of the compound alone to be able to apply it  
21 through a flow through system would be huge.

1           What my university would charge me, depending on what the  
2           compound was -- chemical waste, to get rid of the volume of  
3           contaminated water that you would generate, what the flow through  
4           rack and systems that I know of that would be adequate would cost  
5           alone.

6           So, for example, the way we operate in our laboratory, when  
7           we order one of those mouse boxes you see, and it becomes an  
8           atrazine, it becomes red, green, yellow, whatever it becomes, it never  
9           gets used again.

10          Now you are talking about systems where tomorrow it is not  
11          going to be atrazine. It will be metolachlor. You're going to go buy a  
12          whole new system because your system is now contaminated with  
13          atrazine.

14          Those kinds of costs, unless you are funded by industry to do  
15          the work, simply wouldn't -- you know, it should shut any basic  
16          scientist out, because it would make it -- none of your work would be  
17          EPA acceptable.

18          For example, and there might be a lot gained by looking at --  
19          like the assays we developed, we didn't develop for direct application.  
20          It was just in doing science and things like that.

21          So that's one difficulty.

1           The other difficulty is for xenopus, I don't think -- we can ask  
2           Dr. Kelley, but I don't think xenopus -- I don't think they do well in  
3           flow through water.

4           Even if xenopus does, I think you are going to have difficulty  
5           when you try to move to other species.

6           For example, if you remember my diagram moving from the  
7           laboratory model to the comparative studies, you are going to be  
8           really limited because now if you are coming to a species that doesn't  
9           live in streams, doesn't like flow through, now you have to change the  
10          whole conditions and the studies aren't comparable anymore.

11          DR. GREEN: I'm aware of a lot of facilities that are switching  
12          to flow through. And the definition of flow through is kind of  
13          nebulous right now because you can turn the water flow rate down on  
14          these systems to be less than five percent of the total volume per day,  
15          which is barely a trickle, but enough to keep the water quality stable,  
16          the frogs happy. It doesn't bother or stimulate their lateral line and  
17          get them excited.

18          From that aspect of it, it can be quite practical because you can  
19          stack a large number of animals in small rooms and do more  
20          experiments.

21          But I was under the impression -- I think your point is well

1 taken that tank contamination when you are doing pesticide, studies  
2 like this would be a problem in those systems. They might bind to the  
3 plastic or whatever and it would be pretty difficult to get them clean.

4 But for juveniles, is there a reason why you couldn't set up a  
5 very slow trickle through a flow-through system like this.

6 DR. HAYES: My first reaction for a grow-out study is that it  
7 would be even more difficult in terms of the cost and things. Because  
8 for a tadpole, you know it's only going to be a couple months.

9 There are other people who raise amphibians on the panel.

10 DR. GREEN: I just wanted to hear your opinion. You stated  
11 very earlier on that you would discourage the flow-through system. I  
12 wanted to hear it from you why.

13 DR. HAYES: I also think it is unfounded. I think it's  
14 unnecessary. I think the problems that came up with regards to the  
15 current and submitted studies were not related to flow through.

16 I think that -- again, I don't want to keep jumping on it, but my  
17 3000 datapoints show that that's not the case, that you can achieve the  
18 kinds of results in terms of time to metamorphosis, 90 percent  
19 survivorship, metamorphosis in 45 days with a static renewal system.

20 DR. GREEN: Thank you.

21 DR. ROBERTS: Dr. Kelley.

1 DR. KELLEY: Well, I share your prejudices against the flow  
2 through because the tads hate it.

3 If you go into the field in South Africa, we once studied two  
4 related ponds that were on a golf course. One was above the other.

5 All the tads and the juvies were in the one above where there  
6 was no water flow through. All the adults were in the one below.

7 And either the adults had eaten all the tadpoles in the pond  
8 below since they are notoriously cannibalistic or the animals had  
9 segregated themselves out by preference for waterflow.

10 I think issues of water quality are important. There are things  
11 that we have to check. I agree with you about problems of cost.

12 I think there are more important issues than flow through,  
13 however, in the whole thing that have to be grappled with.

14 I return, and you and I have discussed this before, but let me  
15 raise this issue now again in public to the mechanism question. Of  
16 course it is secondary. You want to make sure you have this effect of  
17 atrazine.

18 If you do, you want to know how it does it. Your main  
19 hypothesis is that it's aromatase.

20 Now, in your animals that were treated with atrazine and had  
21 oocytes that yolked up, what are the only known -- what is known

1 about yolking up oocytes? What is the vehicle for doing that? Why do  
2 oocytes yolk up?

3 DR. HAYES: I'm not sure what you are -- they're filled with  
4 vitellogenin.

5 DR. KELLEY: Right. And where does vitellogenin come from?

6 DR. HAYES: Comes from the liver.

7 DR. KELLEY: Right. And the liver is not the gonad. Right?  
8 So it must have been the case that if estrogen caused the liver to  
9 secrete vitellogenin, that it was secreted at some point. Right?

10 DR. HAYES: Yes. But that's in rana, not in xenopus.

11 DR. KELLEY: Oh, no, no. It has been done in xenopus. Over  
12 and over again.

13 DR. HAYES: No, no, sorry. The atrazine yolking of the eggs  
14 was in rana, not in xenopus. Our work has been in xenopus. We have  
15 only just now started the bleeding (ph) the rana, which are a year old  
16 that I told you about.

17 DR. KELLEY: All right. But let me point out, and I will raise  
18 this again, that there are good endpoints for knowing if an animal has  
19 ever been exposed to a hormone. There are good endpoints for  
20 estrogen and there are good endpoints for androgen. And  
21 contemporaneous hormone measurements are misleading. Right?

1           You know at some point X you have Y. But you don't know  
2           what you had in between that might have caused the condition or a  
3           change in morphology that you are seeing.

4           One way to do that is to look at endpoints that are quite well  
5           established as being created by hormones. So vitellogenin synthesis  
6           is one.

7           If your aromatase hypothesis is right, you might expect to find  
8           an increase in vitellogenin synthesis. The harderian gland has been  
9           established by Chieffy (ph) to express male and female specific  
10          proteins that are under control of estrogen. There are androgenic  
11          endpoints.

12          So these can be used, and I would suggest should be used in an  
13          assay system if we're going to go forward with this kind of a study.

14          The other thing I want to point out is that both myself and my  
15          panel colleague to my right routinely measure low but detectable  
16          measures of estradiol in normal old male xenopus. They are much  
17          lower than female levels, but there is some detectable estradiol.

18          Maybe we could get together and go over the  
19          radioimmunoassays. That would probably be useful. Thank you.

20          DR. ROBERTS: Dr. Heeringa and then Dr. Coats.

21          DR. HEERINGA: Dr. Hayes, I would like to follow up on Dr.

1 Green's question, specifically related to the white paper  
2 recommendations on experimental methodology for any future  
3 studies.

4 In your lab protocols with the three tanks or three replications  
5 per treatment and I believe 30 subjects, 30 tadpoles per tank, when  
6 you analyzed results, let's focus on the laryngeal muscle diameter  
7 results, which are a continuous measure, did you do anything to sort  
8 of look at the inter-tank component of variability in that outcome?

9 You actually had charts, a box and stem plots or distributional  
10 plots that showed the variability on that muscle size.

11 Did you decompose that to cross your three replicates?

12 DR. HAYES: Yes. We do treatment by replicate by sex, by  
13 individual --

14 DR. HEERINGA: What component of that total variance, say,  
15 within treatment is attributable to the replicate or specifically to the  
16 tank environment? Do you have any estimates on that?

17 DR. HAYES: If I can't multiply three times and ten and get 30,  
18 I assure you that I can't remember that. Occasionally, not in the  
19 PNAS studies, but we occasionally on different measures get tank  
20 effects. It depends on the species. For example, in bufo, in toads, it  
21 will often be that one cage will metamorphose.

1 I have always suspected that it was the first couple animals that  
2 metamorphose can stimulate the others, whether it's through hormones  
3 in the water or whatever. We have rarely seen that in xenopus.

4 I do know, for example, that there are shelf effects. Even if it's  
5 one foot apart, different shelves metamorphose at different times.  
6 Within a shelf in my laboratory, especially with the rotations, we  
7 don't see position or tank effects.

8 I did show one of those figures when I talked about rotations  
9 and I had animal controls in the left, right, middle, I did show the  
10 individual data. I treated those as individual experiments even, and  
11 did an inova and showed that there was no difference in time to  
12 metamorphosis or size at metamorphosis, whether you were at left,  
13 right, middle, or end.

14 And we do that in every experiment.

15 DR. HEERINGA: Thank you.

16 Just a comment. I think that the data that's present in your  
17 studies and also in the Ecorisk studies on these tank effects are  
18 extremely critical to setting up and designing for any future studies  
19 that would be done.

20 Tank effects, to the extent that they are present, could very  
21 much change assumptions about significance of some of the results.

1 DR. HAYES: I will also admit I'm not -- I know some  
2 statistics, but certainly if other people want to look at things in ways  
3 that I haven't, then that's available to anybody who wants any of the  
4 data.

5 DR. ROBERTS: Dr. Coats.

6 DR. COATS: Just a comment on the flow through possibilities.

7 I have worked with fish and daphnia. They do quite well in  
8 flow through. Daphnia, being a very small crustacean and the fish  
9 fry of fathead minnows or medaka (ph), start out very small. They are  
10 not bothered by the flow through.

11 So I don't think that should be a hindrance physically as far as  
12 -- water quality improvement would be significant, I think.

13 A different question, then. If you have an inverted or if you  
14 have a threshold which perhaps is exceeded at a high dose, is it not  
15 feasible, then, that you could maybe go downward from point one and  
16 create a dose response that would look more toxicological or --

17 DR. HAYES: I have seen something like that. I can't recall  
18 where, but not necessarily.

19 Like, for example, with a hormone's role in ovulation going  
20 down in dose just simply has no effect in part, you know, because  
21 there may be events increasing sensitivities leading up to the final

1 effect. But going down in dose wouldn't give --

2 DR. COATS: But if you are dealing with a population where  
3 different individuals would have different, ostensibly different  
4 thresholds or different sensitivities, you might --

5 DR. HAYES: I would say that's true in the menstrual cycle. I  
6 don't know if Dr. (inaudible) wants to comment.

7 DR. COATS: Just wondering.

8 DR. ROBERTS: Any other questions for Dr. Hayes? Dr.  
9 LeBlanc.

10 DR. LEBLANC: When you began your presentation hours and  
11 hours ago --

12 DR. HAYES: I was trying to get equal time with Novartis,  
13 Syngenta, Ecorisk.

14 DR. LEBLANC: -- you introduced an assay involving a species  
15 that we then didn't hear anything about. There was a strong color  
16 dimorphism as related to hormone treatment. You must have used  
17 atrazine with these animals. How did they respond?

18 DR. HAYES: That statement that was made that we've reported  
19 effects of atrazine on hyperoes (ph) was incorrect. I'm not sure where  
20 that came from.

21 We have done some stuff, we haven't reported it, but we have

1 exposed animals to atrazine. It doesn't -- Nigel, do you want to  
2 comment? It was your data.

3 DR. NORIEGA: I'm Nigel Noriega. I worked with hyperoleus  
4 (ph) doing some of the exposure studies. Atrazine was only done on a  
5 sample of three or four animals, which wasn't enough to do any  
6 statistics, because all of these color dimorphisms for the nonsteroids  
7 were calculated as a percentage.

8 And it was just too few animals to make any comment about.

9 DR. ROBERTS: Dr. Noriega, just to be clear for the record,  
10 your affiliation is University of California, Berkeley? Is that correct?

11 DR. NORIEGA: I am currently a post doctoral student within  
12 the EPA in the laboratory of Dr. Earl Gray (ph).

13 DR. ROBERTS: Thank you very much.

14 Any other questions for Dr. Hayes?

15 Seeing none, Dr. Hayes, thank you very much for coming here  
16 and presenting in detail the results of your studies and your  
17 conclusions and interpretations regarding those and for answering all  
18 of the panel's questions regarding that.

19 Thank you very much.

20 DR. HAYES: Thank you for the opportunity.

21 DR. ROBERTS: It is not my intent to entertain a give and take

1 among investigators who have different opinions about the data. But  
2 since the presentation you have just heard included some pointed  
3 comparisons with Dr. Carr's work, I thought I would offer Dr. Carr the  
4 opportunity, if he is interested, to very briefly comment or make any  
5 clarifications regarding his study or conclusions, if he wants.

6 DR. CARR: Thank you, Mr. Chairman. Jim Carr, Texas Tech  
7 University.

8 There were just a couple things I wanted to clarify regarding  
9 some comments made by Dr. Hayes. I'll be brief. It is getting late.

10 The first has to do with the issue of dose versus concentration  
11 and whether by putting tadpoles in a one liter beaker there was  
12 actually a depletion of the atrazine as suggested by Dr. Hayes to the  
13 point that the actual concentration -- or the doses were very small.

14 The important thing in this type of study is what actually gets  
15 into the animal. And that can be calculated using the  
16 bioconcentration factor. I have done that. I can prepare a short paper  
17 and give that to the SAP regarding bioconcentration factor,  
18 calculations in rana pipiens. That's the only data that we have.

19 But anyway, if you do the calculation using a value of six, and  
20 this is from a paper by Allran and Karasov, if you use a  
21 bioconcentration factor of six, and bioconcentration factor would be

1 defined as the concentration in the organism divided by the  
2 concentration in the matrix at equilibrium, and assuming that during  
3 the critical period of gonad differentiation we had a volume of two  
4 liters of exposure medium and at our highest dose 19.5 micrograms  
5 per liter, which was the actual measured concentration, and assuming  
6 a wet weight of the total organisms of about two grams, which is  
7 actually an overestimation for the animals at that stage of  
8 development, about stage 49, the depletion of atrazine from the  
9 medium would be about 0.6 percent.

10 So that's a relatively insignificant amount of atrazine that  
11 would be depleted making the concentration relatively stable over the  
12 course of our experiment.

13 And in the report, the final report submitted to the EPA, and it  
14 is available to the SAP, we have a graph illustrating atrazine  
15 concentrations over the course of our experiment. And they remain  
16 relatively constant. Although, at the highest concentration, the actual  
17 average measured value was 19.5 micrograms per liter rather than 25.  
18 It was a little bit less than nominal.

19 So we don't think there was depletion of atrazine from the tank.  
20 We think that they were exposed to fairly close to nominal  
21 concentration throughout the exposure.

301

1           And we think what is important is concentration, not dose. It is  
2 very difficult to estimate the actual dose that's getting into a tadpole  
3 that is swimming around in the stuff.

4           DR. ROBERTS: I was going to say unless you do the kind of  
5 measurements that Dr. Green had suggested earlier where you actually  
6 --

7           DR. CARR: Right. There are some data in fish and I think  
8 some preliminary data in xenopus that were presented at SETAC last  
9 year. But the full type of study has not been done. But those data are  
10 available in the report to the EPA.

11           There were also some issues about water quality comparisons  
12 related to our study suggesting that the quality of the water was poor  
13 after the 50 percent change.

14           Certainly, ammonia levels did increase during the course of our  
15 study. We have supplied data on both unionized as well as total  
16 ammonia levels, as well as pH, dissolved oxygen, conductivity, all  
17 those data are available to the SAP and to the EPA. And I would  
18 encourage anybody who is interested to look and reanalyze those data  
19 if they want to see if there is a relationship between some parameter  
20 and water quality.

21           It is difficult, in fact, to compare to the Hayes work because at

302

1 least in the PNAS paper and EHP paper, those data are not available.

2 There were also some implications. There some quotes  
3 suggesting implicitly that data might be withheld in some way from  
4 our study or other studies to the EPA. I just wanted to remind  
5 everybody that all of our data from all of the studies that the atrazine  
6 Ecorisk panel has performed are available to the EPA. They were  
7 made available by February 28th. They are available to all the SAP  
8 members. They are available to EPA. And they are there for you to  
9 conduct your own analyses if you would like to.

10 You may reach different conclusions based on that, but I would  
11 encourage you to take a look at those raw data if you feel so  
12 motivated. But I wanted to refute the implicit suggestion that there  
13 was some type of data withheld or incomplete data sets.

14 That's all I have.

15 DR. ROBERTS: Thank you, Dr. Carr.

16 Dr. Matsumura.

17 DR. MATSUMURA: Why did you drop the one point at the 25  
18 microgram per liter? You disregard it. That's a big question. You  
19 really stand behind the data or you don't.

20 DR. CARR: That was another issue. Thank you for reminding  
21 me.

1           One of the other issues had to do with calling something a weak  
2 trend. Everybody here has a copy of the ETC paper. You can look in  
3 the paragraph where we report the P value for the correlation between  
4 intersex and discontinuous gonads, and the word weak is not even  
5 mentioned in that paragraph. Especially, in relationship to the  
6 correlations. And I think everyone here has the paper. They can look  
7 for themselves.

8           One of the things that was done in the analysis was to see what  
9 component of the data set was contributing to the trend. The data that  
10 is reported in the ETC paper as well as in the final report that was  
11 submitted to EPA and to the SAP contains the analysis for the whole  
12 data set.

13           One of the things that Dr. Sielkin did in his report which was  
14 submitted as an addendum to our report was to see if the trend  
15 continued at doses below 25 parts per billion.

16           In that particular analysis, the 25 part per billion dose was  
17 dropped out to see if the trend continued amongst the other  
18 concentrations. Now, given two or three data points, of course, you  
19 are not standing on very stable ground in terms of the correlation.

20           But the data that are in the ETC paper and in the final report  
21 have to do with the correlation for all of the data, for all of the doses.

1           That report should have been made available. It can be made  
2 available. That's Dr. Sielkin's independent statistical analysis of our  
3 data. And that would include all of the Cochran armitage tests, as  
4 well as all the other correlations and other analyses.

5           DR. ROBERTS: Are there any other just real quick  
6 clarifications for Dr. Carr?

7           DR. GREEN: What was the big announcement or finding that  
8 was alluded to in that quote from the e-mail that you said was --

9           DR. CARR: At that time and looking back, I probably should  
10 have realized that I would have regret doing something like that, but  
11 there was a huge data set that was getting ready to be submitted to the  
12 EPA. And there was a lot going on. I had 12 or 13 different studies  
13 that were being prepared. That is what I was referring to in that  
14 particular quote.

15           (Thereupon, the time was 5 o'clock p.m.)

16           DR. ROBERTS: Thank you, Dr. Carr.

17           We have more public comments to come. Let me suggest that  
18 we take a short break. 10 minutes or so. And then reconvene and we  
19 will continue with public comments.

20           (Thereupon, a brief recess was taken.)

21           DR. ROBERTS: I'm hoping we can get through the public

305

1 comments by 6:30. If we cannot, we will go ahead and adjourn at  
2 6:30. So for those of you who are thinking about transportation and  
3 dinner and so forth, our tentative plan is to adjourn no later than 6:30,  
4 hopefully, having completed the majority or all of the public  
5 comments.

6 During the break, a question arose as to the availability of some  
7 studies.

8 Dr. Skelly, do you want to pose that question? I think there are  
9 some folks here in the audience who can answer the question for us.

10 DR. SKELLY: I guess I will initially address my question to  
11 Dr. Hayes.

12 Dr. Hayes, you mentioned that you did a study and submitted it  
13 to the Syngenta sponsored Ecorisk group that preceded the ones that  
14 were published in PNAS. And I wondered if you could share that with  
15 the SAP.

16 DR. HAYES: If I could share what?

17 DR. SKELLY: Share the paper.

18 DR. ROBERTS: Is there a report from that study that could be  
19 examined in the docket, entered into the docket?

20 DR. HAYES: The studies that I did, there was one 98XLATZ1.  
21 That was a study where the feeding wasn't appropriate, and we

306

1 terminated the study because of high mortality. I prepared a final  
2 report. I believe it was signed off on by Syngenta Ecorisk.

3 We then followed that with two food studies, 98XL Food 1 and  
4 2, it was called. Those were submitted as final reports. And then we  
5 submitted -- I submitted another large study, 99XLAZT2, which was a  
6 study that examined gonads' growth, weight, development, time to  
7 metamorphosis, larynges, and a final report -- several final reports  
8 were submitted for that starting in 1999, I believe.

9 But can I provide it? I don't know exactly -- I have been given  
10 a letter by Syngenta that I can discuss whatever is necessary with the  
11 EPA involving my involvement. But I don't know if I can give you  
12 those documents directly. I don't know what the law --

13 DR. ROBERTS: Maybe we can ask a representative --

14 DR. SKELLY: I believe we just got it, a package that was on  
15 our chairs.

16 DR. ROBERTS: I'm not sure that that's what we were asking  
17 about.

18 Let me ask someone from the Ecorisk group if there are copies  
19 of those reports that could be placed in the docket?

20 Just to clarify, Dr. Hayes, there were a number of Ecorisk  
21 reports from studies from other members of the Ecorisk group that had

307

1       been entered into the public docket and were available for panel  
2       review prior to this meeting.

3               And during the discussions in this meeting, the mention was  
4       made of these other studies that you had done. And I don't think  
5       those were included among the studies that were on the docket. So we  
6       were just asking if you had them.

7               DR. HAYES: I have copies of them. I don't know if they are  
8       the property of Syngenta, Ecorisk. I know there were a bunch of  
9       studies in 1999 like the fish studies and things that were turned in.  
10       But I don't know the status of those.

11              DR. ROBERTS: Then we can ask someone from the Ecorisk  
12       group about the status of the studies.

13              I think Dr. Sielkin is here perhaps to answer that question?

14              DR. SIELKIN: This is Dr. Sielkin. I'm clarifying that the  
15       packet that is in front of you is the packet that I submitted that had to  
16       do with the analyses that were referred to on laryngeal size, which  
17       was the final report 99XLATZ.

18              It was the final -- it was really a draft report, but it was the last  
19       "final" report that was received. It was the one for which I reviewed  
20       the statistical analyses and a copy of that report as I saw it is  
21       provided to the panel.

1 DR. HAYES: What would be the difference between a draft and  
2 a final? I submitted several final reports, I don't know what happened  
3 to them, on those data.

4 DR. SIELKIN: This is the study final draft -- that's why I was  
5 hesitating with the word final draft. If you submit several final  
6 drafts, then I don't know what to call it. But this is the one that Mr.  
7 Noriega signed off on on 6/23/00.

8 DR. HAYES: No, I mean, there were several reports that -- for  
9 example, I submitted a report. And then you would say increase the  
10 sample size a little bit. Then I submitted another report. I don't know  
11 what happened -- I have copies of those, but I don't know if the  
12 registrant -- I don't know what the law or rule is about me making  
13 those available.

14 DR. ROBERTS: Let's inquire with the Ecorisk group some  
15 more then.

16 DR. BENZ: My name is Katherine Benz. And I was the quality  
17 assurance officer for the projects that are sponsored through the  
18 Ecorisk panel. I'm a consultant to the Ecorisk panel.

19 To answer the question of what is a final report, we did these  
20 studies in the spirit of the good laboratory practices. So for those of  
21 you that are familiar with the good laboratory practices, quality

1 assurance officers tend to look over your shoulder a lot.

2 And they are difficult to do in university settings. We spend a  
3 lot of time in training and writing protocols that we continue  
4 throughout all of the Ecorisk sponsored research, including protocol  
5 amendments and deviations.

6 But as part of that, we also did standard operating procedures.  
7 We did independent quality assurance inspections during the progress  
8 of the studies to ensure that the studies were being conducted.

9 At the end, we did a final report inspection of the raw data. In  
10 this case, as part of that, there is a sign off for the good laboratory  
11 practice statement as well as the quality assurance statement.

12 And we never signed off on the quality assurance statement  
13 because we never got a satisfactory response to some of the quality  
14 issues that were brought up in Dr. Sielkin's review.

15 So we, in fact, never finalized that report. It was submitted as a  
16 draft. And I believe that Dr. Sielkin's report was presented to Dr.  
17 Hayes at a meeting in San Francisco -- excuse me, at Berkeley with  
18 the understanding that we would -- those anomalies or errors in  
19 statistics or in the spreadsheets themselves would be addressed,  
20 corrected, changed, explained and a final report would be reissued  
21 with a satisfactory quality assurance statement.

310

1 I don't know if that answers the question.

2 DR. HAYES: I sent it back to you twice.

3 DR. BENZ: I don't know that we have ever gotten a statement  
4 to the best of my knowledge that the anomalies in Dr. Sielkin's report  
5 were addressed.

6 DR. HAYES: I've got a Fedex receipt.

7 DR. ROBERTS: That's fine. We were interested. It appears  
8 that there is some indeterminant status at least within the Ecorisk  
9 group regarding that report. But we were just curious if that was  
10 something that we could -- a report that we could obtain a copy of  
11 and enter into the public docket so that we could examine.

12 Dr. Kelley.

13 DR. KELLEY: Can we get the feeding reports too? Those  
14 sound like very useful pieces of information.

15 DR. HAYES: I don't know whose property those are. Of  
16 course, I have copies of both feeding studies and final reports that I  
17 submitted, but I don't know if I have the right -- I don't know whose  
18 property those are. I'm not a lawyer. I don't understand legal  
19 contracts.

20 But I have copies. If I get permission, I can turn all the final  
21 reports that I prepared for Syngenta over.

1 DR. MCFARLAND: I'm Janice McFarland with Syngenta. All  
2 of the information we had received from Dr. Hayes has been provided  
3 to EPA and also was provided to the SAP. We definitely allow him to  
4 release any data that he might think is confusing under the property of  
5 Syngenta to you.

6 DR. KELLEY: But we never got this report that he just  
7 described, nor did we ever get the feeding reports. And we still don't  
8 have them.

9 So if you could find them and provide them to us, that would be  
10 helpful.

11 DR. MCFARLAND: I believe that draft report was copied on  
12 the CD that was provided to the SAP. We can check on that, though,  
13 and provide a status.

14 DR. KELLEY: I'll check my CD. But it wasn't in the printout.

15 DR. ROBERTS: Thank you, Dr. Hayes.

16 DR. HAYES: My pleasure.

17 DR. ROBERTS: We next have scheduled a public comment  
18 from Mr. Scott Slaughter on behalf of the Center for Regulatory  
19 Effectiveness.

20 Is Mr. Slaughter here?

21 Just as a heads up, the next person I have scheduled is Mr. Jere

1 White on behalf of the Triazine Network.

2 MR. SLAUGHTER: Thank you very much. I'm Scott Slaughter,  
3 and I represent the center for regulatory effectiveness. It's late. I  
4 will try to be as succinct as possible.

5 CRE's primary interest in this proceeding is compliance with  
6 the Data Quality Act. The Data Quality Act requires among other  
7 requirements that EPA's conclusions regarding atrazine's effects on  
8 amphibian be based on tests that have been demonstrated to be  
9 reproducible and on data that is transparent. And by reproduceability,  
10 that includes interlaboratory reproducibility.

11 The current data base flunks both those tests. No one has  
12 demonstrated that their test results regarding atrazine's effects on  
13 amphibians, if any, have been demonstrated to be reproducible by  
14 other laboratories.

15 To the best of our knowledge, all the tests to date have been  
16 solely one laboratory test. And no one has sent the exact test protocol  
17 to another laboratory to be replicated to see if they get the same or  
18 essentially the same results.

19 In regard to transparency, the data, the relevant data has not  
20 been available to all members of the public. For example, it was not  
21 available to CRE. CRE filed a Freedom of Information Act request

313

1 with EPA seeking essentially all data, all records within EPA's  
2 possession that refer or relate to the amphibian effects of atrazine, if  
3 any.

4 This went several months ago when it was filed. Recently, EPA  
5 responded by saying that Dr. Hayes has submitted seven data sets to  
6 EPA regarding this issue.

7 And Dr. Hayes, after review and consultation with EPA, had  
8 only agreed to release five of those data sets to CRE. We got the five  
9 data sets last week. One of those sets was encrypted. And I have very  
10 few virtues. And one of those virtues is not decoding encrypted  
11 computer disks. So I don't even know what's on that.

12 Based upon Dr. Hayes' testimony today as I understand it, he is  
13 willing to provide to anyone who wishes it and calls him all the data  
14 he has that is relevant to this issue. We will be calling you next week  
15 when you get back to Berkeley to get the missing data and all other  
16 data you have on this.

17 DR. HAYES: I'll be in Brazil next week.

18 MR. SLAUGHTER: Leave a number.

19 That was really it. Once again, I want to emphasize the  
20 importance of this SAP. I don't know whether you are all familiar  
21 with the new Data Quality Act and EPA's data quality guidelines.

314

1           It is late. If you want to ask me questions about it, I will be  
2           glad to bore you with citations to the Federal Register and the Code of  
3           Federal Regulations. But my guess is the answer to that question is  
4           no.

5           Comments to the SAP, we discussed this issue at some length.  
6           We also gave citations on the internet where you can obtain a copy of  
7           our Data Quality Act petition on the atrazine environmental risk  
8           assessment.

9           And on that petition, it was not only CRE, it was also the  
10          Kansas Corn Growers Association and the Triazine Network.

11          EPA's response to the -- the Data Quality Act allows interested  
12          persons to petition EPA or any other affected federal agencies to  
13          correct information that the agency has disseminated which the  
14          persons believe do not comply with the Data Quality Act standards.

15          Once again, one of those standards is reproducibility for  
16          information of this type. And that means interlaboratory  
17          reproducibility and also test validation.

18          One of the critical requirements of test validation is that the  
19          test, lab test on an animal be able -- be demonstrated to be  
20          reproducible among different laboratories. One laboratory runs it.  
21          The other laboratory does the same test until they come up with

315

1 essentially the same result.

2 It was our understanding then and it is our understanding now  
3 that that test and criterion has never been satisfied for the atrazine  
4 database, especially with regard to atrazine's effects, if any, on  
5 amphibians and also on the whole aromatase induction issue.

6 This SAP, it may be the first one to address test validation  
7 issues under the new Data Quality Act and under EPA's new data  
8 quality guidelines. I congratulate you for having that honor.  
9 Actually, I may sympathize with you for being in that position.

10 But I want everyone to be aware here at EPA that you are  
11 operating on a new set of standards in terms of scientific information  
12 being disseminated by EPA.

13 Once again, based upon EPA's white paper, based upon what  
14 we, CRE, knew about the data base before the white paper, and based  
15 upon what we heard at this panel so far, at least one of those -- well,  
16 assuming that we get the data, the transparency test may be satisfied.  
17 But the reproducibility test has not been.

18 And consequently, if this proceeding, if the atrazine review  
19 proceeds any longer or goes forward at EPA, then I think a first step,  
20 a critical first step in order to get out data that is both scientifically  
21 reliable and legally reliable is to make sure that you got validated

316

1 tests on atrazine's effects, if any, on amphibians and also on the  
2 whole aromatase induction issue.

3 Thank you very much. I'll be glad to try to answer any  
4 questions you have.

5 DR. ROBERTS: Any questions for Mr. Slaughter? Anyone  
6 want to discuss the Code of Federal Regulations with Mr. Slaughter?

7 MR. SLAUGHTER: I wouldn't recommend it at this point in  
8 time -- or any other, for that matter.

9 DR. ROBERTS: Thank you very much, Mr. Slaughter, for your  
10 comments.

11 My apologies to Mr. White. I misread the schedule. The next  
12 presenter is Dr. Angelina Dugan with Crop Life America, and then we  
13 will take Mr. White.

14 DR. DUGAN: Thank you.

15 First of all, Crop Life America thanks the EPA for the  
16 opportunity to address the SAP. As many of you know, CLA  
17 represents the manufacturers, formulators and distributors of plant  
18 science solution for agriculture and biotechnology.

19 We believe that the resolution of the question evaluation of  
20 potential developmental effects of atrazine is of great importance to  
21 the determination of environmental endocrine effects for various

1 chemical substances.

2 Overall, the development of robust testing methodology and  
3 interpretation of environmental endocrine cause and effects has  
4 proven to be technically quite complex.

5 More often than not, there has been a lack of consensus on  
6 studies, designs, interpretation of research results and  
7 reproducibility of data within and between laboratories.

8 All of this I might add is not much different than what the panel  
9 is discussing within the current proceedings. And I make this  
10 observation not to criticize any researcher or laboratory, but just to  
11 state the issue.

12 While the lack of concordance fuels lively academic debates,  
13 which I enjoy myself, the outcome may still not resolve or inform  
14 regulatory issues.

15 The Food Quality Protection Act and Safe Drinking Water  
16 (inaudible) stipulated that the test for estrogenic and other endocrine  
17 effects be fully validated as a means of ensuring reliability,  
18 consistency and the data quality for risk assessment purposes.

19 CLA urges the panel to develop the product of their  
20 deliberations and research recommendations in light of the same  
21 technical standards and regulatory necessities.

1           Our trade association has long supported the development of a  
2           validated scientifically sound screening and testing program to  
3           evaluate potential adverse endocrine effects in wildlife and mammals  
4           through both the Endocrine Disruption Screening and Testing  
5           Advisory Committee and, more newly, the Endocrine Disruption's  
6           Method Validation Subcommittee processes.

7           I personally served on the EDSTAC and have continued to work  
8           with EPA and the EDMVS technical experts to see the process  
9           through.

10           The EPA Office of Science Coordination and Policy is  
11           responsible for both the SAP and implementation of the endocrine  
12           screening and testing program. In CLA's opinion, there is an overlap  
13           in the activities of this SAP with many of the same technical issues  
14           and problems that the EDMVS is currently facing.

15           We believe that the efforts of both forums could benefit by the  
16           sharing of EDMVS information from the EPA office of Science  
17           Coordination and Policy and others who are involved in the EDM  
18           process.

19           I point out Professor Gerald LeBlanc who is also a member of  
20           the EDMVS and the SAP and also Dr. Les Touart (ph), who I believe  
21           is in the audience. He's responsible at EPA to develop, in particular,

1 the amphibian endocrine screens and perhaps amongst others.

2 For example, EDSTAC had recommended development of an  
3 amphibian metamorphosis assay to evaluate the frog thyroid axis as a  
4 screen for potential mammalian development effects.

5 Unfortunately, the protocol demonstration of this assay, which  
6 do consideration of several frog species and methodology, has proved  
7 to be problematic for a variety of technical reasons.

8 An alternative assay has since replaced the frog metamorphosis  
9 as a mammalian developmental assay, but its evaluation as a wildlife  
10 screen is still under current investigation by Dr. Touart. And at the  
11 recent June 5th EDMVS, it was communicated that the protocol  
12 demonstration of a frog wildlife assay is projected for December  
13 2004.

14 CLA stresses that the technical difficulties and delays  
15 experienced by EPA and contract laboratories point out critical needs  
16 for not only continued research in the area of amphibian development,  
17 but also follow on safeguard, such as validation, to ensure clear  
18 information for either a regulatory decision or additional endocrine  
19 testing.

20 CLA also emphasizes to EPA and the panel that it's premature  
21 to draw any conclusions on the disruption of aromatase as a potential

1 human health and wildlife issue for atrazine or other environmental  
2 chemicals.

3 EDSTAC had also recommended the development of aromatase  
4 as an invitro mammalian assay to assess the ability of environmental  
5 chemicals to inhibit the enzyme. While there has been good progress  
6 in developing an aromatase screen, the assay is not yet available,  
7 since the initiation of the interlab validation phase is still several  
8 months away.

9 As far as I know, there have been no efforts to date to research  
10 aromatase uptake as a potential wildlife screen.

11 In closing, CLA supports thorough evaluation of research  
12 recommendations to determine potential environmental development  
13 effects on amphibians and mammalian species. However, we do not  
14 believe that the issue of potential developmental effects has been  
15 resolved for atrazine or potentially given the state of the science can  
16 be resolved for other environmental chemicals as well.

17 CLA urges the panel to uphold the laboratory and field data  
18 reproducibility and test validation as standards and conditions for  
19 implementing amphibian developmental assays for risk assessment  
20 purposes.

21 Thank you. I wish you well in your deliberations.

1 DR. ROBERTS: Thank you, Doctor. You jumped right into  
2 your comments. Can I ask you for the formality of introducing  
3 yourself for the record?

4 DR. DUGAN: Sorry. Angelina Dugan. I'm the director of  
5 science policy for Crop Life America.

6 DR. ROBERTS: Thank you. Let me check and see if the panel  
7 has any questions for you. Apparently not. Thank you very much.

8 Mr. White, who will be followed by Dr. Fawcett.

9 MR. WHITE: Mr. Chairman, we actually have four people who  
10 will be part of this presentation.

11 DR. ROBERTS: Thank you. If I could ask each of you to  
12 introduce yourself before you speak for the record.

13 MR. WHITE: Mr. Chairman, members of the committee, my  
14 name is Jere White. I'm the executive director of the Kansas Corn  
15 Growers Association, Kansas Grain Sorghum Producers Association  
16 and also serving in kind of an ad hoc capacity, certainly an unpaid  
17 capacity, as chairman of the Triazine Network.

18 A little bit about the Triazine Network. It was formed back in  
19 1995 as somewhat of a response by growers of over 30 commodities,  
20 and certainly, that many states, to provide a vehicle for participation  
21 in the US EPA special review of the triazine herbicides. And that

1 certainly is atrazine, but also simazine.

2 Our objective is to ensure that EPA has and utilizes the best  
3 science. That's why we participate in events such as today.  
4 Membership encompasses farm groups from border to border and  
5 certainly sea to sea as well.

6 The executive committee is composed of farm organizations  
7 from Kansas, Missouri, Florida, California and Hawaii. We're a very  
8 diverse group.

9 atrazine has been used as the foundation of our weed control  
10 programs since the 1950s. It has been around for a long time. It is a  
11 very important product. And we know this product well.

12 We know how to steward it in a way that provides safety for  
13 ourselves, or at least we believe we do, and also for the environment  
14 that we farm and, probably more importantly, that we live in. We  
15 have confidence in the product.

16 I must say that I will diverge from some of my written  
17 comments to respond to a few things that we have seen over the last  
18 few days. One of the things that really struck me was at the end of  
19 Dr. Hayes' presentation when he placed the slide but he didn't discuss  
20 about a corn yield increase of 1.2 percent.

21 I'm sure in lieu of the discussion that had taken place the

323

1 previous four or hours, it was meant to minimize the value of that.

2 Let me clarify what that means. Number 1, the 1.2 percent we  
3 don't agree with. But it is, if you think in terms of percentages,  
4 maybe a fairly insignificant number.

5 But on Kansas Farms, at the farm gate, it is equivalent in corn  
6 and sorghum makers to about 120 million dollars a year. That's per  
7 year at the farm gate. That's 120 million dollars that's available to  
8 support education, medical, ambulances. It is real dollars at the real  
9 level. And quite frankly, that is significant where I come from.

10 I think also it was placed up in the context if you looked at the  
11 tail end of the presentation right before then, it was in the context of a  
12 lot of different issues that are certainly not any issues to EPA in a  
13 special review. Again, we're coming up on the nine year anniversary.

14 These issues are not new to EPA. Certainly, not even new to  
15 the SAP. Previous SAPs have dealt with many of the studies that were  
16 laid up as a part of this frog functioning as a canary in the mind type  
17 situation.

18 Many of the studies actually have been addressed and, to some  
19 extent, minimized from the original assumptions in the study based on  
20 review of previous SAPs.

21 In fact, the position of EPA today is that atrazine is not likely

1 to be a carcinogen in humans. I don't think you would have taken that  
2 away from the tail end of the previous presentation.

3 Also, I must comment that I was taken back by the picture of  
4 the unhappy frog. I don't know if the frog is happy or not. It was  
5 obvious that it was a little bit different type of frog or in a different  
6 condition. I wouldn't deny that. I'm not sure what that means. I'm  
7 not a frog guy. I don't know these things.

8 But it just suggested that this was representative of perhaps  
9 unhappy frogs in the field, which you would expect after 40 years of  
10 usage. And yet, I really didn't hear the case made for that. In fact,  
11 I'm not sure I heard a very strong case made for why the very  
12 profound laboratory observations and even the specific field  
13 observations that were shown and very eloquently presented to the  
14 panel, how we could even have frog populations -- the term robust  
15 was used earlier, I don't know if I'm qualified to use that term, but  
16 how we could have even surviving frog populations if this was, in  
17 fact, a legitimate situation in the field.

18 And that is an issue to growers. All throughout this process, we  
19 have seen situations where models have been applied to suggest  
20 impacts out in the real world. It's been tough to explain this when I  
21 go home and talk to my growers how these situations can exist and yet

1 they go out behind their house and in the farm ponds that are  
2 surrounded by corn and see very, to use the term, robust frog  
3 populations and certainly not seeing a lack of frogs or other types  
4 wildlife that would be associated with that type of healthy population.

5 I was also taken back by the link visually between atrazine and  
6 DDT. Kind of brings me back to my earlier days in the 60s when  
7 everybody was reading Silent Spring. And certainly, I don't mean to  
8 minimize the influence of the work of Rachel Carson or certainly the  
9 impact of DDT. But I will tell you atrazine is not DDT. I guess I  
10 hope I shouldn't need to tell you that. I'm taken back by that kind of  
11 sensationalism.

12 I guess another thing that hits me, here we are, it is another  
13 SAP. And guess what, we had another press release come out  
14 yesterday on low sperm counts in Missouri folks that had been  
15 exposed to atrazine.

16 Not necessarily new data, but we got a new press release. It has  
17 been a while since we have really seen that. I guess we had one at the  
18 technical briefing time frame from Dr. Hayes. It was work that had  
19 been around since the previous November and presented at SETAC,  
20 but all of a sudden it was all over the world news. You could get hits  
21 -- a tremendous job of selling the news. Whether it is news worthy or

326

1 not, I guess that's for others to decide.

2       Again, thinking of this new study from Missouri, I haven't had a  
3 chance to really look at in detail, but it is interesting to me that the  
4 males that were considered viable candidates for the study were  
5 partners of pregnant women. That's how they were selected. And yet  
6 the study is about low sperm count. I don't know if that makes male  
7 men in Boone County, Missouri nervous or not, but I would think it  
8 ought to.

9       To some extent, part of the problem that we see is that the  
10 studies conducted by Dr. Hayes in our opinion have led to the SAP.  
11 Wouldn't have gotten much more than a snicker if they would have  
12 been submitted by a registrant or some other person in the same  
13 fashion.

14       I'm pleased to see that there is a very openness about the data  
15 that was suggested today. But I'm also aware of the situation that Mr.  
16 Slaughter talked about earlier where when the data was finally  
17 released under a Freedom of Information Act request, it was still  
18 encrypted.

19       If you look at the white paper prepared by the agency itself on  
20 Page 17, they talk about for these latter studies reviews were less  
21 detailed because EPA did not have access through the study office for

1 the full range of raw data.

2 You wouldn't assume that based on the presentation this  
3 afternoon. In fact, you saw the shelves with reams of data that were  
4 available to anyone that really had an interest in it.

5 The last time there was a public meeting that talked about this  
6 issue and the agency during the technical briefing April 16th of last  
7 year, there was actually a little bit of disagreement whether data was  
8 available at that point because it was being referenced in the technical  
9 briefing.

10 In fact, the director of special review and reregistration did  
11 clarify to everyone in the audience that they did not have data at that  
12 time.

13 Again, it is good news to hear that it is available now. We  
14 expect to be seeing it. But it is also interesting that when the cameras  
15 are rolling, the data is readily available, but the historic perspective  
16 of this is that the data has not been available. And I think you can  
17 attest to it. I don't think you find much of it in your packet.

18 Dr. Hayes told an audience at Duke University last January in  
19 talking about -- I suppose he was looking at the potential for the  
20 interaction of different products that he talked extensively about this  
21 afternoon.

1           He used, and I have a quote here, that with other pesticides, the  
2 other pesticides "act like frog bullies because they hold them down  
3 and let atrazine beat them up."

4           And I guess my observation is when you are struggling with  
5 making your case with good data and good work, at least shared data,  
6 you try to give the herbicide in this case an evil personality. And I  
7 have a problem with it. I think we have done it a little bit in trying to  
8 suggest a link in the same sort of effects between atrazine and DDT.

9           I would hope that that would be thoroughly discussed by the  
10 panel.

11           Well, in Kansas, it appears that the frog bullies have not been  
12 very successful because the frogs seem to be doing very well. I don't  
13 mean to be too facetious, but the implications that seem to be negative  
14 to a pesticide always have a certain amount of implied truth or  
15 believability about them. After all, how could anything that has been  
16 used on American farms for 40 years still be good. How could it even  
17 be effective.

18           Well, trust me, there is plenty of competitors in the weed  
19 science industry that are looking for alternatives to atrazine. If they  
20 were truly out there in all the ways you measure alternatives, they  
21 would be used by American farmers.

1 I guaranty you, one of the companies that would love to have an  
2 alternative to atrazine that was really functioning that way would  
3 probably be Syngeta. They would be several million dollars ahead.  
4 They would be selling a higher priced product at the end of the day.  
5 They wouldn't have to deal with the continual types of issues that they  
6 have had to.

7 I'm very happy that they have taken it upon themselves to  
8 provide the science with all the deficiencies that have been talked  
9 about. They have done more than any other registrant I can envision  
10 ever would for any product that is out there.

11 That's part of my concern as someone representing production  
12 agriculture is that if you can't make the case with the kind of science  
13 that's been provided on atrazine, what product do you think would  
14 ever sustain itself against the types of continuous allegations that are  
15 out there?

16 Now, to be sure if there is a problem, farmers want to know  
17 about as much as anyone. They are the ones that apply the product.  
18 They are the ones that apply it on where they live by and large. So  
19 they have an issue in this, a very serious issue. But they also need to  
20 know that sound science carries today, and I think that's what this  
21 panel is about.

1           Most of the times when we testified about EPA positions, we  
2           unfortunately have not always been in agreement. Usually, at the end  
3           of the day we're getting pretty close. And although it is close to the  
4           end of the day now, the issue is not.

5           I would say that we do think EPA basically got this right.  
6           There are confounding conclusions to be drawn. I guess I almost  
7           liken it almost a he said she said debate, and it continues even at this  
8           hour, where comments are made by one person and then the other  
9           person feels like they need to redeem themselves. This could go on  
10          forever.

11          And I think the EPA's position to get established standard  
12          protocols is the appropriate way to do thing. Not only is it the legal  
13          thing to do if they think there is an issue with effects on amphibians,  
14          they don't have a foundation to make regulatory decisions on now, and  
15          I know that that's not so much the issue of the panel, but I think you  
16          can do a lot to help EPA get this right. Because getting it right is  
17          critical. Not just for atrazine, but for all the other products that are  
18          out there.

19          Once atrazine is off the radar screen, other products of course  
20          will continue to move forward. And quite frankly, unless we want to  
21          see agricultural production move out of this country, we have to come

331

1 out with some systems that really can establish the safe use or the  
2 unsafe use of those products and move on to others.

3 Today, I brought three of my comrades up here to help also  
4 raise some other issues that they have focused on. With me here  
5 today is Stephanie Whalen, president of the Hawaii Research Center,  
6 Bill Kubecka, doctor of veterinary medicine from Texas, former  
7 president of the National Grain Sorghum Producer Association,  
8 current president of the Texas Grain Sorghum Producers.

9 We originally had a farmer from Boone County, Missouri, that  
10 just happened to be from Boone County that was here. He had to  
11 leave. I don't know if he has gone back to get his semen checked, but  
12 he did have to catch a flight earlier. So we have pitch hitting for him  
13 Gary Marshall, my counterpart from the association from Missouri.

14 Now I ask for Stephanie Whalen to speak first.

15 DR. ROBERTS: Before we go any further, I think the panel  
16 wanted to ask you a question.

17 Dr. Kelley.

18 DR. KELLEY: So in the interest of total disclosure, I should  
19 tell you I come from an agricultural family myself. My family grows  
20 cranberries.

21 And I know that one of the things that we always think about in

332

1 growing crops is we're always looking down the line to the next  
2 herbicide or pesticide because regulations change.

3 Now, in some countries, I understand in Europe, atrazine is not  
4 used. I think even in Switzerland where Syngenta is headquartered. I  
5 wondered if you are aware of what farmers in Europe used for their  
6 broadleaf weeds in place of atrazine and whether we had tried that  
7 here in America.

8 MR. WHITE: I think the European system is just so much  
9 different. It is not based -- it is buy and large, if I understand it  
10 correctly, which I maybe do or don't, it is based mainly on a level of  
11 detection.

12 There are some situations, for instance, France has proposed a  
13 ban of atrazine for corn, but not for grapes. I guess it depends on who  
14 has the political power there.

15 I do understand that there are alternative herbicides that are  
16 used that are not, how should I say, they are very similar, in fact,  
17 might even be azine but they are not atrazine because of the political  
18 climate that has changed that situation in Europe. But it used in some  
19 places. It is not a European ban.

20 But there are some -- some countries like Germany, I believe,  
21 initiated more or less a level of detection in groundwater standard.

333

1 And because of that, they have moved to other products.

2 And they also because of those changes are not as competitive  
3 in the world market in the production of their commodities. That is a  
4 fact.

5 DR. KELLEY: Thank you.

6 DR. ROBERTS: Let's move on with Ms. Whalen's presentation.  
7 Welcome.

8 MS. WHALEN: Stephanie Whalen. Today I'm speaking on  
9 behalf of our research center, what is formerly known as the White  
10 Sugar Planters Association. Just to let you know, I'm representing the  
11 sugar industry. We are the research and support organization for that,  
12 the Hawaii industry for the last -- over 100 years.

13 We have cooperated with the government agencies at the  
14 federal and state level in health and environmental studies. And we  
15 have a long history of interacting in the regulatory process. In fact,  
16 we have been involved in the registration of pesticides even before  
17 EPA was formed.

18 Scientists from our organization were involved with the early  
19 work with the triazines. Because the Hawaii soil is different, we did  
20 plant and soil metabolism studies for the triazines for our industry.

21 I just also wanted to indicate, which is not in the handout

334

1 received, that we also instituted a voluntary water monitoring  
2 program prior to the time that EPA set up the MCL level at 3.

3 And just to give you a feel for how seriously we take  
4 stewardship of the products that we use -- and because we exist, our  
5 industry existed on four of the major islands and over 200,000 acres,  
6 we felt that it was incumbent upon ourselves to do that work even  
7 though it wasn't required at that time.

8 I won't go over everything I had to say. Some of it is duplicate  
9 of what Mr. White has said. Though, I do want to stress the fact again  
10 that atrazine is one of the most widely researched compounds,  
11 herbicides in history of pesticides.

12 And like you said, if we can't move forward with atrazine, we  
13 don't think the science will ever be there for any compound.

14 Based on our history of experience with the compound, the  
15 sugar industry and the other growers when we formed the Triazine  
16 Network, we entered into this process with some level of familiarity  
17 of use and time, but also with the open mind that there may be some  
18 unknown adverse effects out there and we really need to know about  
19 that.

20 We wanted to be sure that the best science would determine that  
21 and that we were committed to accepting whatever the science

335

1 reveals.

2           After all, as Jere pointed out, it we the farmers and our  
3 families that are on the frontline of any exposure. And it is our lands  
4 that are going to be contaminated first.

5           So we really are committed to the results of sound science, but  
6 based on reasonable demands without political intervention, free of  
7 scientific turf battles or special interest agendas.

8           I think it's of some value for you to understand the process  
9 which began, that Jere alluded to, back on November of 1994. And  
10 just to give you some kind of idea of the processes we have been  
11 following, this panel is a brief but very important part of the  
12 continuum from that time.

13           So far the process has primarily focused on atrazine as was  
14 said. But we have been through in this process rumors, information  
15 leaks, drafted documents, scientific advisory panels, proposed  
16 documents, interim documents, administrative changes, numerous new  
17 studies, new laws, the food quality protection acts which came in the  
18 middle of this, totally upended the transparent regulatory review  
19 process that we started, proposed new cancer guidelines that are  
20 seemingly now caught up in bureaucratic quagmire which does affect  
21 this process, a lawsuit which allows a single party to dictate the

1 process, public scares generated by activists and the press, and now  
2 scientists' challenges of each other.

3 We have watched various players, mainly the government, come  
4 and go. We've heard about many speculated health effects. More  
5 recently, our listening to speculation on ecological effects.

6 Through all of this, it has been an experience for us, an  
7 experience in which we continue to have faith that in the end sound  
8 science will prevail through the efforts of impartial experts such as  
9 yourselves and the panel that was heard from yesterday. However, the  
10 speculation on human or environmental effects and the timing of the  
11 public releases have not ceased to amaze us, and their end does not  
12 appear to be on the horizon.

13 We thought the evaluation and the speculation of the sprague  
14 dawley female rat hormone system and its significance for the  
15 potential human cancer risk was finally settled after three scientific  
16 advisory panels were convened, 1988, 1995, 2000. And now it  
17 appears that a fourth will be convened next month on the same issue  
18 driven by the NRDC.

19 We patiently listen and try to fully comprehend the details of  
20 the rat endocrine hormone system and its relevance or lack thereof to  
21 the human population as we have for the last two days listened

1 intently to the recent amphibian concerns.

2 We have asked for assistance from experts. In their rat work,  
3 we asked for Canton Health Sciences International to help explain  
4 this to us and then to present testimony for us.

5 In that process, we were upset to find that some of the data  
6 from reports that were being used in an earlier document were not  
7 available for our experts to review.

8 It's again troubling that the initiating raw data, this is a subject  
9 that has come up already, that has generated many of the studies  
10 reported on yesterday and today were generated, the need for this  
11 scientific advisory panel, have been difficult or next to impossible to  
12 access. And hopefully, as Jere has indicated and Dr. Hayes has  
13 indicated that that data will be fully available.

14 The inability to access raw data is particularly disconcerting to  
15 our organization. And the reason that is is that several years ago we  
16 were involved in cooperating with the EPA sponsored study  
17 contracted to third parties in which we participated through split  
18 samples and providing them access to our industry.

19 The analytical results were not similar. Although, we  
20 voluntarily produced our raw data to the agency, they were never able  
21 to get the raw data from the party they contracted with, but EPA

1 published a government report with questionable results anyway,  
2 never acknowledging there was industry collaboration and that there  
3 was a discrepancy in the split samples.

4 Our follow-up investigation discovered the laboratory involved  
5 had no previous experience in pesticide analysis, and was not required  
6 to report raw data in their contract.

7 I mention this because of the similarity of the current situation  
8 and the inherent problems with deciphering and reproducing methods  
9 in the open literature. And the fact that EPA has not proposed a  
10 validated test system to study is also problematic.

11 It was not clear yesterday from the various comments that the  
12 members of the panel made -- and I'm still not sure that people are  
13 clear on the differences on studies conducted under good laboratory  
14 practices, because if you have never been engaged in one, you really  
15 don't know what it requires, and those appearing in refereed journals.

16 The purpose of good laboratory practice was to improve the  
17 ability to reproduce the study, totally reproduce the study, without  
18 having the bodies around to ask at any future date.

19 So if you have reports that are GLP like, and I don't know  
20 exactly what that was supposed to mean, but GLPs are formal  
21 regulations that require the submission of all raw data and data audits.

1 It's like a financial audit, you have auditors of a financial institution,  
2 by a nonparticipating party.

3 I also want to clarify. Scientists don't get certified. I have  
4 been involved in GL -- that's what we do a lot of. But we are required  
5 to document proof of training. It's the studies that must meet the GLP  
6 standards and have quality assurance statements as was indicated  
7 before showing any deviation that occurred in how it might affect the  
8 results of the study.

9 I believe -- there is a big difference in reports from the  
10 literature, not reports, but studies or papers from the literature which  
11 seems to be referred to versus reports that I think we're talking about  
12 GLP like reports.

13 I believe that GLP regulations and complying to that provides a  
14 level of confidence way over the peer review system. What bothers  
15 me is why the agency does not apply at least a minimum level of these  
16 types of requirements to data from reported studies that generate  
17 significant concerns such as what we have here today.

18 I did point out a problem that I saw in the report entitled  
19 atrazine Induced Hermaphroditism at 1 ppm in American leopard  
20 frogs. I'm not going to go through that. You can read about that. It's  
21 really something -- it was very factual data that was recorded

1 incorrectly and that bothers me in terms of how it influences the rest  
2 of the validity of the information, though it might be just being  
3 careless.

4 It bothers me also that this process I described continues to  
5 generate endless speculation. First related to the sprague dawley's  
6 endocrine peculiarity and now the amphibian endocrine systems.

7 As scientists, you know a phenomena can be studied for the  
8 length of a career with tens of graduate students and many post docs  
9 looking at every conceivable hypothesis. Each study leads to more  
10 interesting questions to explore. That's great. That's what science is  
11 about. And I enjoy that discovery process myself.

12 However, I think it is important to remember that this is part of  
13 a regulatory process, which is expected to be somewhat more  
14 pragmatic. There should be a point at which the exploration ceases  
15 with a reasonable assurance that a sound decision based on the weight  
16 of evidence will be made.

17 That doesn't preclude that continued discovery and appropriate  
18 revisiting of decisions doesn't happen.

19 It appears from yesterday's discussion, if I understood it  
20 correctly, that this process could embark on a whole new area of  
21 research, an area in which endpoints, baselines and protocols, and

341

1 there seems to be a difference of opinion there, have yet to be  
2 defined, because I have heard that some endpoints are very definitely  
3 established in the literature, and yet I've also heard that there seems  
4 to be some concern. So an area also that seems to be open to many  
5 approaches and potentially years of study.

6 Those of us, and we are the consumers, we are the consumers of  
7 this extremely useful product, are looking to you, a panel of  
8 prominent scientists, to make decisions based on the existing weight  
9 of evidence.

10 We can all speculate on better ways to redo the less than perfect  
11 studies. But how much more data are really warranted? How relevant  
12 or significant are the data to the viability of the amphibian  
13 populations which have existed in these environments for over four  
14 decades and which are now facing reduced exposures through  
15 voluntary rate reductions and stewardship programs developed over  
16 the last eight years.

17 We do appreciate the difficulty of your task and we thank you  
18 for your willingness to assist the agency in moving forward on this  
19 issue. I thank you for this opportunity.

20 DR. ROBERTS: Before we move to the next presentation, let  
21 me ask the panel if they have any questions for you. I see none.

1 Thank you very much for your comments.

2 Let's move on to the next individual.

3 MR. KUBECKA: My name is Bill Kubecka. I am a family  
4 farmer from Palacios, Texas. We grow sorghum, rice, cotton and  
5 cattle. Again, as Jere previously mentioned, I have served as  
6 president of the National Grain Sorghum for two years and currently  
7 serve as the president of Texas Grain Sorghums Producers. I am also  
8 a veterinarian by education and training.

9 As a farmer, I value the importance of atrazine. Despite  
10 intensive research by weed scientists and makers of competitive  
11 products for over 40 years to identify atrazine alternatives, the use of  
12 atrazine in herbicide programs continues to provide benefits at a  
13 relatively low cost.

14 But even more important, research shows that without the use  
15 of atrazine, yields in our grain crops will drop regardless of the cost.  
16 atrazine is the most significant herbicide, especially in conservation  
17 tillage programs, and use about 90 percent of those acres.

18 A point that I would like to bring up and that's grain sorghum is  
19 not corn. We face a difficult task in grain sorghum in that we are a  
20 smaller crop, much smaller crop, about somewhere less than 10  
21 million acres versus corn of 80 million.

1           Therefore, there are fewer, substantially fewer weed products  
2 available to produce sorghum and certainly fewer alternatives  
3 regardless of the cost or the effectiveness.

4           I think the last time we looked at EPA, we had one product in  
5 line to be registered for sorghum. That's all. That's insecticide,  
6 herbicide, everything. It's a big issue for us in sorghum even at 10  
7 million acres.

8           atrazine is the cornerstone of our weed control options. If there  
9 are real issues in our health and environmental effects, I think this  
10 has been pointed out before, we need to know about them.

11           I use this product where I live, where my kids, my grandkids  
12 live. But I must respond to the reliable information in order to  
13 operate my business and be a steward of my land and family.

14           As a veterinarian, I know that proper care requires a proper  
15 diagnosis. And in this case, we don't even know if there is a problem.

16           It is the position of Triazine Network, and I concur, that this  
17 issue should be partitioned away from other issues being dealt with in  
18 the completion of reregistration of atrazine.

19           The agency with the advice and counsel of the SAP should work  
20 to approve a protocol and initiate a call-in to help in the further  
21 investigation of these alleged issues.

1           If they believe further investigation is warranted, the EPA  
2           should not draw any conclusions at this time despite attempts from  
3           activist groups to regulate on the assertion of these studies.

4           Only one needs to remember previous excursions involving the  
5           toxic soup theory I think that was proposed by Tulane, the Cornell  
6           monarch butterfly scare and, most recently, the University of  
7           California, Berkeley, GMO corn issue in Mexico, to realize that  
8           sound science will survive the test of substantial review. But using  
9           preliminary science to regulate is not in the best interest of the  
10          regulated or the regulator.

11          DR. ROBERTS: Thank you, Doctor. I don't see any questions.  
12          Let's move ahead.

13          MR. MARSHALL: Good afternoon. My name is Gary  
14          Marshall. I am the CEO for the Missouri Corn Growers Association.

15          Unfortunately, Terry Hilgadick who is a board member of mine  
16          was to be here this afternoon and offer this testimony. But his time  
17          ran out. He had to catch a flight back to, as Jere said, central  
18          Missouri.

19          I hope that I can adequately postulate some of his remarks as  
20          well as add in a few of my own. But I'm going to try to be very, very  
21          brief in my remarks.

1           We have actually three different companies. We have a grower  
2           organization, which is kind of a lobbying group, we have a marketing  
3           group and we have an environmental group, all nonprofits that work  
4           for the Missouri Corn Growers Association. I work as their CEO  
5           there.

6           In addition to that, I have over 30 years of experience in  
7           agricultural products and background. I live on a farm. I currently  
8           operate a farm. And in a previous life, I applied thousands of pounds  
9           of atrazine on thousands of acres of corn in a liquid fertilizer  
10          operation that we had for about 15 years.

11          So I do have some, in fact, probably some extensive background  
12          in using the product in central Missouri area.

13          In the nine years since the special review began, more than 200  
14          studies have been conducted on the safety and benefits of atrazine and  
15          have been submitted to the EPA. Question after question about the  
16          safety of atrazine to humans and the environment has been answered  
17          in a timely fashion by the registrant and growers through the use of  
18          sound science.

19          I would like to add here. I'm really pleased to have the  
20          opportunity to participate in this whole process as a grower. It has  
21          been nine years now, because I also served, along with Jere and

1 Stephanie, on the executive committee of the Triazine Network, nine  
2 long years that we have been dealing with this issue.

3 But we're hoping that it finally is beginning to come to some  
4 sort of conclusion, we hope.

5 Over four decades of on-farm use of atrazine has been a very  
6 reliable indicator of atrazine in the production of corn, grain  
7 sorghum, sugar cane and other crops.

8 The health and environmental effects of the triazine herbicides  
9 have been more carefully studied than any other pesticide group.

10 Obviously, I'm not a scientist. But I do understand some of the  
11 benefits of having atrazine as a tool for crop production on our  
12 particular farm where we raise corn, soybeans and we also have cattle.

13 In Missouri, we have over three million acres of corn. We use  
14 atrazine on over 70 percent of that corn, or about 2.1 million acres.

15 Atrazine allows for good weed control, allows Missouri  
16 growers to utilize conservation tillage practices on the overwhelming  
17 majority of the corn that we grow today. That has changed a bunch in  
18 just the last five to ten years.

19 But this helps eliminate or even reduce plowing of fields for  
20 weed control. Makes cropland then less vulnerable to soil erosion in  
21 some cases by as much as 90 percent.

1           Jere mentioned the cost differential and what it means. In  
2           Missouri, we figured that cost differential. To switch to another  
3           product, you have two problems. You have a switching cost to go to  
4           another product, and there is a significant cost to it. And secondly,  
5           you have a yield drop or a yield cost. And we calculate that to be  
6           over \$20 per acre in Missouri.

7           If you figure that on our 70 percent of the acres that's treated,  
8           that's over 42 million dollars a year. If you want to look nationwide,  
9           it is a billion dollar a year difference at a minimum for corn farmers.  
10          That's just corn farmers.

11          It's a significant big product that we use. We use a lot of  
12          pounds of it. The loss of this product to some of the smaller grower  
13          community would be even as staggering probably as it would be to the  
14          corn community.

15          Missouri farmers are really dedicated to an ongoing proactive  
16          approach, to environmental stewardship. And in fact, in Missouri we  
17          have what we call the WRASP program. The Watershed Research and  
18          Stewardship Program was initiated in 1999.

19          Since that time we been collecting data on watersheds, and we  
20          have best management practices to producers studying the various  
21          management practices that we can utilize in a cost-effective manner to

1 help keep these products on the fields where they belong and not onto  
2 water systems.

3 Since that time the data has been collected in two large  
4 watersheds in Missouri encompassing over a million and a half acres  
5 of watersheds. And it is really interesting to note as far as the EPA is  
6 concerned these two watersheds are now being proposed to be delisted  
7 from the 303 impaired water list for the state of Missouri, the 303 D  
8 list of impaired water.

9 So we think that's very significant, and we're hopefully going to  
10 be able to take these practices then and move them into other areas of  
11 the state and perhaps across the country to help again make our  
12 products more effective where they need to be and more  
13 cost-effective.

14 Regarding the frog populations in our area, again, I'm not a  
15 scientist, but on my farm we have rivers that run -- one river runs  
16 through the farm. Another river runs on the south side of the farm.  
17 We have wetlands, one that I have built as a conservation reserve  
18 program. We have other wetlands that preexisted there. We have  
19 springs, ponds, we have wells.

20 I can tell you just from my observations the frog and amphibian  
21 population appears to be doing very, very well. In Missouri we have

1 noted no decrease in population that I'm aware of. Nothing has been  
2 noted to us by the University of Missouri, the State of Missouri, the  
3 Departments of Conservation of Natural Resources.

4 And, in fact, the Conservation Department is actively pursuing  
5 as we have been for a number of years a frog season which in  
6 Missouri starts about two weeks from today. So with that, I do hope  
7 that your process that you are involved with here does make a  
8 difference. Because we are counting on you to work with the EPA  
9 registrant and all interested parties to make sure that the information  
10 that is presented is of value, it makes sense and we want to move this  
11 process forward.

12 With that, Mr. Chairman, I would thank you for this  
13 opportunity.

14 DR. ROBERTS: Thank you very much for your comments, Mr.  
15 White. Is there anything else you or your group would like to say?

16 MR. WHITE: Thank you to the panel for your time. I know it  
17 has been tough. I'm back there where I can get up and move around,  
18 but it has to be merciless up here.

19 Thank you again.

20 DR. ROBERTS: Thank you, Mr. White. Dr. Kelley would like to  
21 follow up on a previous question.

1 DR. KELLEY: This is a question directed towards Syngenta  
2 and also towards Dr. Hayes. I did not see in these Ecorisk Syngenta  
3 documents that we were provided as printouts originally the printout  
4 of one of the early reports from Dr. Hayes that was supported by  
5 Syngenta. And that is on the CD.

6 I had not read it until this hard copy was just provided to me.  
7 But on the CD I could not find the feeding data. If you could provide  
8 those to us, one of you, that would be great. Is that possible?

9 DR. ROBERTS: Dr. Mcfarland, did you want to respond to  
10 that?

11 DR. MCFARLAND: Thank you. I did find in -- for those of  
12 you who might want to look for that draft report on the disk that was  
13 supplied to the Scientific Advisory Panel, it is in the miscellaneous  
14 report section. And that's where that is located.

15 We did have with us a CD of all the raw data and information  
16 that Dr. Hayes provided us on that study that was submitted to EPA  
17 previously, and we have it with us here, so we'll print that off and we  
18 will be happy to provide it.

19 When I was scanning through it I wasn't seeing the feeding,  
20 though. Anything we have we'll definitely provide. I'm sure Dr.  
21 Hayes will be happy to provide that report too.

1 DR. KELLEY: So the report we have been provided with is a  
2 report that was signed off by Dr. Noriega in June of 2000, I think. Is  
3 that what it is June of 2000? So that's the one we have been given on  
4 our disk. Is that the last one that was received from Dr. Hayes?

5 DR. MCFARLAND: Yes, that was the last report Syngenta  
6 received from Dr. Hayes.

7 DR. KELLEY: Also accompanying this report are a series of  
8 critiques from the statistical consultant, and those are 2002.

9 DR. MCFARLAND: I believe that was just a summary done in  
10 2002. The statistical report that Dr. Sielkin discussed with Dr. Hayes  
11 in his lab were discussed back in 2000. and I think that's the date of  
12 the larger report attached in your hard copies there.

13 DR. KELLEY: Dr. Hayes, in this report you actually looked at  
14 sex ratios. In the report you state that there is no effective atrazine  
15 on the sex ratios that you observed. And yet in your next study you  
16 did get an effect

17 Could you comment on this discrepancy?

18 DR. HAYES: Yes, in the report --I'm not sure which report you  
19 are talking about.

20 But in the bigger study that I did, the successful study that I  
21 did we reported no effects on sex ratio, however, there were several

1 animals that were noted in the data that were question marked or that  
2 were marked for review.

3 And these are animals that did turn out and have the same  
4 gonadal abnormalities. I have pointed that out to members of the  
5 panel. We didn't fully appreciate the extent of the gonadal  
6 abnormalities until about November. It would have been after that  
7 report was turned in.

8 DR. KELLEY: In that report you state those were animals that  
9 died before stage 66 and they were excluded from the analysis. I  
10 mean that's what it says here I just read it.

11 DR. HAYES: I would have to look at the report and see, but  
12 there's -- even on the slide I show, you can see in the sex column from  
13 that data set there are animals marked for further review for comment  
14 or animals that were question marked.

15 DR. KELLEY: In this initial study, the mortality appeared to  
16 be greater than that which you have in your current study. So you had  
17 mortality up to 24 percent.

18 I think in the -- you had significant mortality associated with  
19 ethanol. You did analyze survivorship and there was a mortality in  
20 the estradiol treated group of up to 24 percent, but also in the other  
21 groups as well.

1 Do you have a feeling for why your mortality has come down  
2 since or what was going on with that initial study?

3 DR. HAYES: I don't recall that. I have to look at what study  
4 you have. The study that I recall where there's high mortality, there  
5 was initial study in 1998 where we got high mortality, we terminated  
6 the experiment, then we went back and then did the two food studies,  
7 which we filed reports on. And then there was a fourth study.

8 I would have to look at those data to see which study you are  
9 looking at.

10 DR. KELLEY: Okay. Thank you.

11 DR. ROBERTS: Thank you, and thank you, Dr. Mcfarland and  
12 Dr. Hayes.

13 Dr. Fawcett, I believe is the next speaker.

14 DR. FAWCETT: I'm Richard Fawcett. I'm here representing  
15 the Iowa Corn Growers Association. I appreciate the opportunity to  
16 appear before this panel to share a few issues from an agriculture  
17 perspective relative to atrazine.

18 This panel has been charged with the primary task of  
19 examining the hypothesis that atrazine could directly affect  
20 amphibians. It's also been suggested that atrazine through its known  
21 mechanism of action of inhibiting the photosynthesis could cause an

1 indirect effect on aquatic ecosystems by reducing algae and plant  
2 growth. I want to look a little bit at that issue.

3 If, in fact, atrazine were to have a negative impact on an  
4 aquatic ecosystem through reducing photosynthesis of algae and  
5 aquatic plants, that means we would have to have currently a deficient  
6 level of plant growth.

7 Quite to the contrary. We're looking at a lot of monitoring  
8 data that I will quickly share with you. In the conclusions of EPA's  
9 Office of Water, the problem we have with the vast majority of waters  
10 in the atrazine use areas, the corn belt primary, our problem is of  
11 having much too much aquatic plant growth, not too little.

12 Looking at the 2000 national 305 B report, excessive nutrients  
13 are listed as the most common pollutant affecting lakes, reservoirs,  
14 and ponds, accounting for 50 percent of the impaired waters. The  
15 other pollutants in order of their occurrence were metals, siltation,  
16 total dissolved solids, oxygen depleting substances and last is  
17 pesticides.

18 In the past we haven't had numeric criteria for nutrients to  
19 measure impairment of waters. To try to help with that situation, a  
20 couple years ago EPA published eco-regional nutrient criteria. We  
21 have also had Regional Technical Assistance Groups or RTAGS that

355

1 have been looking at the data and developing their own suggestive  
2 standards.

3 The upshot of this is is by the year 2004 states must adopt  
4 enforceable standards for total nitrogen, total phosphorus and  
5 chlorophyll-a as a measure of plant growth

6 When you look at those proposed standards and compare it to  
7 current monitoring data, which we can see is really throughout the  
8 atrazine use areas, the nitrogen, phosphorus, and chlorophyll-a  
9 concentrations are routinely two to four times those EPA criteria or  
10 the numbers RTAGS have come up with.

11 For example, if you look at Iowa, we don't have 10,000 lakes in  
12 Iowa, but I guess we have about 131, those were monitored  
13 intensively throughout a year. All but one of those 131 lakes  
14 exceeded the proposed standards for nitrogen and phosphorous, with  
15 some being 20 fold above the standards.

16 Looking at chlorophyll-a, 98 of the 131 lakes exceeded the  
17 chlorophyll-a standards.

18 We have really too much aquatic plant growth in that region,  
19 not too little.

20 Just very quickly, to look -- this is a chlorophyll monitoring  
21 data. The standard is that line way down almost at the baseline. You

1 can see the vast majority of those lakes, the 131 there have far too  
2 much algae growth at least as far as the aquatic ecologists are  
3 concerned.

4 Quickly, we can look at the nutrients, the cause of that  
5 excessive plant growth and of course the cause -- the detrimental  
6 effect that excessive nutrients have is to cause excessive plant growth  
7 which then can lead to low oxygen or hypoxia as the plant material  
8 degrades or metabolism can reduce oxygen.

9 These are the nitrogen numbers. You can see that all except  
10 one of those lakes far exceeded the nitrogen standards, some with --  
11 tremendous. You see that variability. I think it is one of the possible  
12 confounding factors in field studies. I really believe in the field  
13 studies with these kind of differences in water quality can certainly  
14 have an impact.

15 That shows the phosphorous concentrations in the lakes.  
16 Again, all except one far exceeding the proposed standards.

17 Excessive nutrients and/or excessive aquatic plant or algae  
18 growth are common causes listed as impairments for 303 D lists. For  
19 example, in Illinois, 57 percent of impaired waters list excessive  
20 nutrients and/or algae as the cause of impairment. In Iowa, 50  
21 percent of the impaired lakes list excessive algae as the cause of

1 impairment.

2 Again, we have excessive levels of aquatic plant growth, not  
3 too little. Where do all these nutrients come from that cause these  
4 impairments?

5 Part of it in our region where we are we have very fertile soils.  
6 I think we  
7 may have had higher levels of nutrients in our waters than maybe  
8 some of the ecologists believe in the past.

9 Certainly, agricultural practices can increase the loss of  
10 nutrients. The fertilizers we use, the products you put in the land can  
11 increase losses of nutrients into those surface waters.

12 Farmers have been active for years in trying to reduce nutrient  
13 losses. Both for economic and environmental reasons. They have  
14 adopted a lot of practices to try to reduce nutrients and stop this  
15 excessive  
16 algae growth. This may be things like conservation tillage that was  
17 mentioned earlier.

18 And of course, by the way, atrazine is really one of the most  
19 important tools that let's us use this system. They may be  
20 conservation buffers, even putting in wetlands. We heard about that  
21 from Gary just a minute ago. Wetlands being designed to try to

1 denitrify nitrate.

2           So farmers understand they have to be as efficient as they can  
3 and try to reduce nutrient losses.

4           But there is a great fear with these standards being set extremely  
5 low that farmers may be asked or forced into making even greater  
6 reductions that might reduce their bottom line, that might cost more  
7 money, reduce yields, and reduce their incomes.

8           Because with the EPA Office of Water, the message they are  
9 getting from that organization is they need to reduce nutrient losses  
10 dramatically to reduce the growth of aquatic plants and related  
11 problems like hypoxia.

12           We have almost an opposite message that they may be getting,  
13 depending, I guess, how some of this turns out. And if we look at the  
14 IRED, there is a low tier risk assessment by EPA that suggests that  
15 atrazine in surface water at least could hypothetically reduce harmful  
16 or cause harmful reductions in aquatic plant growth, and that we need  
17 to stop that.

18           And that conclusion there is we don't have enough plant  
19 growth. We need more.

20           Well, when a farmer sees those varying messages, they can  
21 easily ask the question which one do you want. Which one is true.

1 They both can't be.

2 I think EPA will have to be careful to make sure there is a  
3 consistent basis and a sound basis for their recommendations.

4 One other issue to quickly hit on ponds here, farmers have  
5 constructed thousands of farm ponds across the country that have  
6 really changed the landscape. Most of these ponds have been  
7 constructed with federal cost sharing money.

8 In fact, in Iowa, NRCS says over 80 percent over the years of  
9 ponds put in have relied on federal cost sharing money. And while  
10 there are very important secondary uses of these ponds, recreational  
11 uses, fishing, swimming, that type of thing, in order for that pond to  
12 qualify for cost sharing, by law, NRCS must certify that its primary  
13 purpose is one of the three things there. Often, it's all three. Grade  
14 stabilization for erosion control, flood control or water quality  
15 protection of downstream waters.

16 By design, these farm ponds are constructed at sites that are  
17 vulnerable to runoff. Their primary purpose, if you get government  
18 cost sharing, and than more than  
19 80 percent of them, the primary process is to trap and process runoff  
20 from agricultural land. Runoff that has sediment, nutrients and  
21 pesticides.

1           The presence of a compound like atrazine or a nutrient in a  
2           pond rather than being measure of impairment is a measure that is  
3           doing what it was designed to do. These were placed there to catch  
4           that runoff process and hopefully increase degradation and protect  
5           downstream waters.

6           Farmers are concerned that they have taken land out of  
7           production, paid their part for these, they may be penalized in the  
8           future regulatory standards are applied to those ponds.

9           We are almost to the end here. These kinds of structures are  
10          very commonly used in watershed protection projects. Here is an  
11          example from southern Iowa, Lucas County Lakes Water Quality  
12          Protection Project.

13          The town of Sheriton (ph) uses three reservoirs. As you can see  
14          on the top Lake Morris, Lake Ellis and Red haw as their drinking  
15          water source. That watershed was evaluated, and NRCS designed a  
16          number of structures to protect that water source.

17          Every one of those little red triangles there is a water and  
18          sediment control basin, kind of a miniature little pond. The dotted  
19          lines show what we would more maybe traditionally think of as farm  
20          ponds.

21          And if you look carefully, you will see some little blue circles

361

1 with a little fountain coming out of the top. Those are wetlands,  
2 constructed wetlands at the tops of those reservoirs.

3 There is more than 50 of those of kind of structures that have  
4 been put into that landscape. Again, farmers are concerned, will they  
5 benefit in the end or will they be penalized for putting them in.

6 They do help in protecting that water quality of the reservoirs.  
7 But also as a side benefit, they provide aquatic habitat. By the  
8 construction of those thousands of farm ponds across the country, we  
9 have created aquatic habitat where there wasn't any before, helping to  
10 replace some of the wetlands that are lost because of development or  
11 agriculture.

12 And another thing, just from personal experience, we have heard  
13 before, but they are full of frogs. I don't know what -- a robust  
14 population either. But there is a lot of frogs in those ponds.

15 From personal experience, I can relate -- on my own home farm  
16 in eastern Iowa, we constructed a farm pond almost 20 years ago with  
17 government cost sharing money. The thing that surprised us about  
18 that pond was the very first year it was full of water. Throughout the  
19 whole year we had an unbelievably high population of bull frogs. That  
20 pond was surrounded and still is by corn fields treated with atrazine.  
21 The frog population remains there.

1           We have the last overhead. You can applaud for this,  
2 conclusions, just in summary, I appreciate being here to talk to you on  
3 a busy day. I have tried to talk fast and get through this.

4           But in conclusion, algae and plant growth in waters in the  
5 regions where atrazine is used is excessive. It's not suboptimal. It is  
6 very unlikely that atrazine would have a detrimental effect in these  
7 areas by reducing aquatic plant growth when we are trying to reduce it  
8 already by reducing nutrient losses.

9           And in fact, atrazine is a critical tool in the systems we use to  
10 try to reduce nutrient, sediment and pesticide loss.

11           Most farm ponds were designed by NRCS to trap and process  
12 farm runoff that includes nutrients, sediment, and pesticides. And  
13 again, the presence of those compounds in a farm pond shouldn't be a  
14 measure of impairment, but a measure of their effectiveness.

15           And lastly, those farm ponds that we have put on the land have  
16 created aquatic habitats where there were none before, and at least  
17 anecdotally they have a lot of frogs in them.

18           Be glad to take any questions if anybody has any.

19           DR. ROBERTS: Thank you, Dr. Fawcett.

20           Are there any questions regarding his presentation? I don't see  
21 any. Thanks very much for coming and sharing your comments and

363

1 views with us.

2 Well, earlier today, I confidently proclaimed that we would  
3 complete the public comments today, but later today I said we  
4 wouldn't take any new public comments after 6:30 in view of not  
5 getting the panel too rundown.

6 With that in mind, let's close the session today. There are a few  
7 people that are registered to present public comments. I thank them  
8 for their patience. And ask them to come tomorrow morning. We will  
9 convene at 8:30. We will continue and complete, I say with utter  
10 confidence now, the public comments tomorrow morning and then  
11 we'll begin our deliberations.

12 A question in the back?

13 MR. HEDBERG: I would beg the patience of the panel to take  
14 five, about five minutes of their time.

15 DR. ROBERTS: Are you one of the public commentators that was  
16 scheduled?

17 MR. HEDBERG: Yes.

18 DR. ROBERTS: If it would be a hardship to present tomorrow  
19 and your comments are short, we can take them tonight.

20 MR. HEDBERG: I would certainly appreciate that.

21 DR. ROBERTS: I don't think that will be a problem at all. Go

1 ahead and come forward if you are ready now and introduce yourself  
2 to the panel.

3 MR. HEDBERG: First of all, I thank everybody for your  
4 patience. I do have to leave town tomorrow.

5 My name is Rob Hedberg. I am the director of Science Policy  
6 for the Weed Science Society of America.

7 My comments, the Weed Science Society is pleased to be here  
8 regarding this assessment on potential effects of atrazine on  
9 amphibians.

10 WSSA is a nonprofit organization of academic research,  
11 extension, government, industry, scientists committed to improving  
12 knowledge and management of weeds in agricultural, aquatic, forest,  
13 horticulture, range, right of way, natural area environments. Together  
14 with our affiliate associations around the country we represent  
15 approximately 4,000 scientists.

16 We're very interested in the special review and re-registration  
17 because atrazine as you have heard plays such a major role in weed  
18 management throughout much of the nation.

19 To preface my comments too our impressions that we're going  
20 to talk about today are really based on review of the white paper. To  
21 the extent that our comments seem critical, it might follow through

1 with what Ecorisk said, that the white paper was somewhat harsh in  
2 its judgement.

3       However, it's important, I think, at this point to be critical,  
4 because the endpoint for the use of this analysis is a regulatory -- it  
5 will result in a regulatory decision. So I think it is appropriate to be  
6 rather harsh in our critique of the studies.

7       We would like to make several comments. To provide a new  
8 perspective on this discussion, we are basically in substantial  
9 agreement with most of the agency's analysis of the studies that were  
10 evaluated, but we're not in full agreement with the proposed strategy  
11 from our perspective.

12       I wanted to start with general comments and then respond to  
13 some of the specific questions posed to the panel. Foremost, weeds  
14 are a very significant agricultural, environmental, and public health  
15 problem, and atrazine is a very important herbicide that has been used  
16 more than any other compound to control these weed problems.

17       It has been used on millions of acres every year for more than  
18 40 years.

19       For most recent years, it has been used on over 60 million  
20 acres annually in this country. It has been used in a number of  
21 different crops, in different weed management systems, under many

1 different environmental and climatic conditions.

2       The sheer magnitude of its use is testimony that this herbicide  
3 has provided and continues to provide enormous benefits to many  
4 different people.

5       Because it has been used so widely and for so long, atrazine and  
6 its impacts are better characterized, documented and understood than  
7 for most other chemicals in the environment. Certainly we know  
8 more about atrazine than we do about any of the alternative herbicides  
9 that may be available.

10       Arguably, we know more about atrazine and its impacts than we  
11 do about alternative weed management practices whether they be  
12 biological, mechanical, cultural type practices.

13       Based on this extensive history, it is only reasonable and  
14 prudent that we should look beyond the laboratory studies to detect,  
15 confirm, repudiate, hypothesize adverse effects. Laboratory analysis  
16 disengaged from real world validation can give misleading  
17 impressions. And this was recently the case when results of  
18 preliminary lab studies on monarch butterflies and bt corn pollen were  
19 extrapolated beyond their supportable scope.

20       A great controversy was created, public anxiety was raised  
21 unnecessarily and, ultimately, the field studies demonstrated that the

1 effects found in the laboratory do not correspond to real impacts  
2 under real conditions.

3 We think it is important, this distinction between effects and  
4 impacts, because it is the crux of determining ecological relevancy.

5 In addition to recognizing the distinction between laboratory  
6 effects and real world impacts, it is important to keep the purpose of  
7 this analysis in mind as we understand it.

8 This is not a human health issue that we have been convened to  
9 look at. We are convened to look at ecological risk.

10 The human health issue has been addressed by other SAPs and  
11 have been, I think, fairly thoroughly considered when the agency  
12 issued its Interim RED earlier in January.

13 These amphibian analyses are being conducted to examine  
14 ecological impacts. And as such, they must be to conducted to  
15 facilitate the risk benefit comparisons which are required another  
16 statute, namely FIFRA.

17 With this in mind, we feel that any future studies and course of  
18 studies as you go forward should be more closely aligned with  
19 finding, verifying, quantifying and comparing impacts under real  
20 world conditions and with testing hypotheses about laboratory  
21 induced effects.

1           In response then to the specific questions posed to the panel we  
2           have the following comments.

3           Comment on the agency's conclusions about the field  
4           experiments inadequacy to ascertain absence of a causal relationship.  
5           In our opinion, the field studies did not demonstrate repeatable  
6           impacts of significant magnitude to be convincing that there is a real  
7           world problem under field conditions. Although this warrants  
8           additional examination, and we think we should keep going forward,  
9           it appears that field impacts do not even begin to approach the level  
10          of concern that would be enough to outweigh the benefits that this  
11          herbicide provides.

12          Especially compelling from our look at the data was the Illinois  
13          field study that found only 2.8 percent with a range of 2.3 to 3.6  
14          percent of "intersex" prevalence over three years of sampling in the  
15          field. That does not seem like an extraordinary environmental impact.

16          Question 3A, comment on the agency's conclusions that the  
17          laboratory studies provide the basis for a plausible hypothesis about  
18          atrazine developmental effects.

19          Our read of this was that the studies at this point do not offer a  
20          good basis for establishing any hypothesis. It appears to our reading  
21          that the studies were plagued with multiple deficiencies and

1 variability.

2           And our conclusion, much like the agency said, is that we have  
3 to some standardized protocols that will yield repeatable results.

4           One of the things that stuck in one of our reviewers minds was  
5 the original Hayes study which triggered this described the containers  
6 as being nondescript plastic containers typically used to house  
7 laboratory mice.

8           Well maybe the question had been looked at. But to us, we  
9 know there are concerns about estrogenic compounds being released  
10 from plastic containers. If you are going to be looking at hormonal  
11 impacts, you should definitely qualify what kind of containers are  
12 being used and how this was considered in the study.

13           So I think what we're saying is that these studies are not at a  
14 level right now to justify the hypothesis that the agency presented.

15           Question 3B. We concur with the agency that the variability  
16 makes it impossible to ascertain a relationship between atrazine  
17 exposure and amphibian developmental effects.

18           Question 6A. Comment on the agency's determination that  
19 there is not sufficient data to reject the hypothesis that atrazine can  
20 cause developmental effects in amphibians.

21           We felt this is a very difficult line of questioning, because it is

1 more subjective than scientific. Will there ever be enough evidence  
2 to prove a negative?

3 In our opinion the question should be is there enough  
4 information to prove that atrazine causes developmental effects in  
5 amphibians. And based on the studies which were reviewed, we think  
6 that answer is no.

7 Question 8A. Comment on the proposed sequence of study  
8 objectives. We fully support the development and validation of  
9 reliable laboratory protocols before any further analysis is pursued.  
10 Subsequently, we agree that the original studies indicating possible  
11 developmental effects must be independently reproduced before  
12 further studies are warranted.

13 If any developmental effects are found in the laboratory, it  
14 would be appropriate at that point to focus further investigation on  
15 carefully designed field surveys that can answer whether or not these  
16 effects occur in the field environment as well.

17 Finally, if effects are found in the field, further study should  
18 focus on determining whether or not there are significant ecological  
19 impacts. Although elucidating the mechanism of any developmental  
20 effects is of scientific interest and we would not like to stop that  
21 pursuit, we think that impact is what is important for further analysis

1 in the regulatory scheme.

2 The final question that we are going to respond to is, comment  
3 on the agency's recommendation that *X. laevis* be used as a primary  
4 model.

5 This is not our area of expertise, but there are some questions  
6 which come to mind right away. There are several concerns about the  
7 species. The first is its relevance to discerning ecological impacts in  
8 North American environments.

9 Using this species as an indicator of possible impacts in North  
10 America would introduce another interspecies variable into an  
11 analysis that already seems plagued by unmanageable variation.

12 Secondly, as documented in the white paper, the species is  
13 already known to have a unique hormonal response as to  
14 environmental variables which differ from the North American  
15 populations.

16 Finally, as an organization, we're very concerned about  
17 invasive species and would not like to see overuse of a species that  
18 might conceivably escape into the environment, such that African  
19 clawed frogs would become the next northern snake head.

20 In closing, what we would like to do, the take-home message is  
21 that WSSA would like to contrast the absolute certainty we have about

1 the benefits of atrazine as a weed management tool and to contrast  
2 that to the current uncertainty and ambiguity associated with the  
3 amphibian risk experiments evaluated by the agency in the white  
4 paper.

5 Although the agency has found the overall weight of evidence  
6 so uncertain that does not support any definitive conclusions  
7 regarding amphibian developmental impacts, we are absolutely  
8 certain that atrazine is a herbicide that provides significant economic  
9 and ecological benefits when compared to the available alternatives.

10 DR. ROBERTS: Thank you, Mr. Hedberg. I think there may be  
11 a couple of questions. Dr. Green has one.

12 DR. GREEN: I just want to make a comment because I would  
13 like you to know that we know and are aware of some of the concerns.

14 I don't want to speak for Dr. Hayes, but concerning the  
15 polycarbonate rat cages that you have expressed an interest in here,  
16 they actually are designed for use in animal experiments. The kind of  
17 plastic that they are does not break down to endocrinological active  
18 metabolites.

19 They were designed for mice because of that exact concern.  
20 Over time, though, those will gradually be replaced. In my  
21 experience, there are containers that are suitable for housing xenopus

1 that are designed for use in storing human food. Currently in our  
2 facility, that's one of the requirements, that if you are going to house  
3 them in something else that is portable, that it is small.

4 There are sources where you can buy the kind of plastic  
5 containers that won't do exactly what you propose they might do.

6 Your concern about *xenopus laevis* being an evasive nonnative  
7 species is a real one. As you know, that happened in the past, in the  
8 early 1980s.

9 Fortunately, those populations that have escaped and gone to  
10 states where they have cold winters and freezing and thawings, as far  
11 as I know, those populations have not thrived under those conditions  
12 and some have disappeared entirely.

13 But as a result of that, it takes a state permit in many states  
14 now to actually keep *xenopus laevis*. And I think they will be even  
15 more increasingly regulated as we go into it. So those things that you  
16 bring up here, those points will be taken into consideration.

17 DR. HEDBERG: Very good. Thank you.

18 DR. ROBERTS: Dr. Denver.

19 DR. DENVER: I just wanted to say that there is some recent  
20 evidence that bisphenol-a can actually be released from polycarbonate  
21 cages. But nevertheless, that should be constant across treatments.

1 So one would have to invoke an interactive effect with atrazine. I just  
2 wanted to say that.

3 MR. HEDBERG: This is definitely very far out of our area of  
4 expertise, but are there other -- could glass containers be used? I  
5 raise this as an outsider questioning the questions that come to my  
6 mind. Are there other devices or procedures that could be used that  
7 eliminate that question entirely from the analysis?

8 DR. GREEN: A big concern is the cleanliness and the ability  
9 to put things through very large autoclaves and cages washers. And  
10 certain kind of plastics don't handle that very well when you do  
11 repeated cleanings.

12 But, yes, there are other types of plastics and other containers  
13 that are designed for animal use that we think, although you never  
14 know, probably don't release any kind of compounds as they degrade  
15 over time that would affect animal experimental results. Glass  
16 containers will work as well.

17 When you have thousands and thousands of animals, though, it  
18 becomes very difficult to manage large glass containers, although  
19 some people still use them. I think with time, though, things will be  
20 replaced to more suitable containers designed specifically for  
21 xenopus.

1 DR. ROBERTS: Dr. Kelley.

2 DR. KELLEY: Just to respond to the glass. Glass is good, and  
3 the frogs like the glass, but steroids stick to glass. So the way you  
4 get around it is you coat the glass with something that prevents them  
5 from sticking, but then you have some new chemical.

6 The problems are endless.

7 DR. ROBERTS: On that note, perhaps we should close the  
8 session. I appreciate, Mr. Hedberg, your willingness to come and  
9 give public comment this evening.

10 Dr. Hayes did you have something you wanted to take care of  
11 before we closed down?

12 DR. HAYES: What has been handed to you is not my final  
13 report. It doesn't include limb deformities that we reported. It  
14 doesn't include snout vent length versus laryngeal regressions that we  
15 included. It doesn't include any of the chemical measurements from  
16 PTROS or any of the data that were attached to the final report. I will  
17 try to track down that.

18 DR. ROBERTS: If you could try and track down that final  
19 report for us, we would be grateful for that. Thanks for clarifying  
20 that.

21 Thanks, Mr. Hedberg, for coming and commenting this

376

1 evening.

2           Thanks to the other public commenters who presented today. It  
3 has been a very useful session, I think, for the panel. We look  
4 forward to continued comments tomorrow morning and beginning our  
5 deliberations. The session is now closed. We will reconvene at 8:30  
6 a.m. tomorrow morning.

7           (Thereupon, the session was recessed at 6:55 p.m.).

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378

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