FIFRA SCIENTIFIC ADVISORY PANEL (SAP)

OPEN MEETING

JUNE 18, 2003

DAY 2 OF 3

Located at: Crown Plaza Hotel
1489 Jefferson Davis Highway
Arlington, VA 22202

Reported by: Frances M. Freeman

CONTENTS

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

Larry, do you have any comments?

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

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

Thank you.

DR. ROBERTS: Thank you, Larry.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Good morning Dr. Bradbury.

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

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

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

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

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

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

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

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

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

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

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

A brief history. Atrazine was originally registered by Ciba.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Now, this mechanism -- I'm sorry you can't quite read that. There is very few small prints on these slides. This mechanism was raised yesterday as potentially applying to the props of the issues we're approaching with the frog. To date nobody has actually assessed that in any -- not assessed it at all.

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

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

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

many reviews.

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

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

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

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

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

and not mediated by ER."

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

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

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

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

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

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

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

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

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

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

background, a very rapidly developing science.

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

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

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

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

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

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

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

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

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

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

there is a Hecker study in rana.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

assessment paradigms evaporate.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

just the scientific process of correlation.

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

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

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

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

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

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

In that paper, Hayes suggests that the problem that he is facing in sites two and three, where there is a very marked difference in hermaphroditism time and identical levels of atrazine, the suggestion is that site two was only intermittent use and that site three it was sustained use of atrazine. I don't know data to support that. There is another speculation in the nature paper that that very high abnormality level at site three was due to run-off from neighboring states because there aren't any farms in that area or wind transfer.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

And likewise there has been one done at the University of

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

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

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

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

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

the issue of what people are calling abnormalities.

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

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

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

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

testosterone into an estradiol, is the favored one.

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

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

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

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

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

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

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

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

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

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

doses.

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

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

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

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

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

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

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

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

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

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

natural populations.

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

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

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

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

Thank you for your attention.

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

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

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

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

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

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

Dr. Kelley.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DR. ASHBY: Right.

DR. KELLEY: Thank you.

DR. ROBERTS: Dr. Green.

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

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

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

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

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

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

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

I just want to get your opinion as a representative from

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

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

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

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

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

generic statement. The science in general is not doing this at the moment and they probably should be. When this got to the point recently, where it was going ahead from SAP we had a meeting with Mel Anderson and the process started of trying to understand this. It certainly needs a lot of detailed studies and the fear is it maybe different for rana, it may be different of a microbism than a field, but the actual trying to get together of one of these modeling compartmentalizations models that Mel Anderson developed -- we started to do -- it was most relevant initially, we were starting a couple years ago to think about the GrNH mechanism and that's where we wanted to know exactly what gets in.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DR. ASHBY: It must be.

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

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

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

DR. LEBLANC: Agreed.

DR. ROBERTS: Dr. Isom.

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

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

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

Probably the most relevant and often the inferential way of

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

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

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

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

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

intrinsic estronicity.

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

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

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

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

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

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

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

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

DR. ROBERTS: Dr. Delorme.

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

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

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

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

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

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

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

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

DR. DELORME: Agreed.

DR. ROBERTS: Dr. Kelley.

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

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

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

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

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

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

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

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

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

DR. KELLEY: Could I raise a question?

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

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

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

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

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

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

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

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

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

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

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

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

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

DR. KELLEY: Thank you.

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

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

discussion today.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

extract of a paper by Wallace.

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

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

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

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

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

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

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

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

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

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

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

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

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

systems is not present.

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

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

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

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

So, we discriminate then between induction versus expression

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

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

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

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

information that he had presented for the gonadal aromatase.

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

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

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

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

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

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

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

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

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

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

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

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

Thank you.

DR. ROBERTS: Thank you Dr. Breckenridge.

I believe Dr. Denver has a question for you.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DR. BRECKENRIDGE: Thank you.

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

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

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

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

DR. ISOM: Thank you.

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

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

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

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

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

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

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

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

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

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

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

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

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

DR. ROBERTS: Dr. Skelly.

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

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

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

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

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

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

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

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

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

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

DR. ROBERTS: Dr. Kloas.

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

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

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

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

Secondly, for the critical dose things are also many reports in the past literature but also a more recent one, you can induce -- not in just male developing xenopus, but you can induce feminization in phenotypic getting more phenotypic of females for being in concordance with the terminology. At 10 minus eight molar and 10 minus 9 molar it seems to be at least the tendency for getting feminization.

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

related and I would like to clarify it more.

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

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

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

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

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

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

DR. ROBERTS: Dr. Kelley.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DR. BRECKENRIDGE: Thank you.

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

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

(Brief Break.)

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

Welcome Dr. Hayes.

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

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

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

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

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

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

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

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

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

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

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

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

involved in to research and to personnel working in my laboratory. So, those are my affiliations, not all of them necessarily contributed to the work that I'll present today.

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

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

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

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

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

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

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

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

In that regard because of not only sensitivity during

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

replicated. That's not true.

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

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

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

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

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

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

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

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

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

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

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

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

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

interfering with androgen action.

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

DR. KELLEY: You mean viability?

DR. HAYES: Viability. I'm sorry.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

metamorphosis in the same section of larynx.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DR. KELLEY: Which replicate are these data from?

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

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

DR. HAYES: Two and three? Yes.

DR. KELLEY: You just described it?

DR. HAYES: Yes.

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

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

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

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

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

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

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

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

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

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

So these ends are actually different in your --

DR. HAYES: No, they are not.

DR. KELLEY: They look different.

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

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

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

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

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

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

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

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

DR. HAYES: Yes.

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

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

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

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

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

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

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

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

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

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

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

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

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

atrazine produced intersexes or hermaphrodites in 16 to 20

percent of the exposed animals.

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

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

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

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

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

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

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

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

problem.

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

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

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

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

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

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

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

blindly.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Questions?

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

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

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

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

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

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

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

DR. ROBERTS: Dr. Denver.

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

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

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

DR. ROBERTS: Dr. LeBlanc.

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

DR. HAYES: Yes.

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

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

DR. LEBLANC: Is it included in here?

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

DR. LEBLANC: Thank you.

DR. ROBERTS: Any other questions?

Go ahead, Dr. Hayes.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Questions?

DR. ROBERTS: Dr. LeBlanc.

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

DR. HAYES: Yes.

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

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

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

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

DR. LEBLANC: It is higher level?

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

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

DR. HAYES: Yes.

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

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

DR. HAYES: What time is it now?

DR. ROBERTS: That would be another 20 minutes.

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

DR. ROBERTS: Yes.

Dr. Kelley and then Dr. Denver.

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

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

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

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

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

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

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

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

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

DR. HAYES: Oh, I'm sorry.

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

DR. HAYES: Thank you.

DR. ROBERTS: Dr. Denver.

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

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

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

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

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

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

DR. HAYES: When we do -- sorry.

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

animals from each tank?

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

default oocyte, as opposed to inducing sperm.

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

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

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

DR. HAYES: Thank you.

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

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

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

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

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

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

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

DR. HAYES: Yes.

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

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

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

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

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

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

personnel in my laboratory conducted.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

discuss in the North Plat, because it is not in the vicinity of corn growing areas. There has been some discussion about the site.

Back in the laboratory, we analyzed, we dissected and analyzed the animals. This is a typical male, that's a typical female. I'm not prepared to say that there are no effects in females. I'm saying with the methodology that we have been using for rana and xenopus, we don't detect abnormalities or and kinds of effects in females in any of the assays that we have used.

So we -- at this point, we have actually -- are only analyzing the males. At least in the assays we are using, we do not detect gonadal abnormalities or hormone abnormalities in females. This is an animal collected from the field. I believe one of the pictures that was in both the Nature and the EHP Paper -- I will below this up again. Very clear, testicular lobules with oocytes. In fact, if you do serial sections, it is not just a few, the entire gonad -- throughout the entire gonad, every lobule have one oocyte. I won't bore you with more pictures, I will just show you where we found hermaphrodites at some percentage. I'll show you the percentages as well.

One of the big surprises was the North Plat River in Wyoming. The site in Utah -- again, there is local atrazine use on the golf course here. These sites are all associated with an area of high atrazine use.

So, that's almost understandable. The one big concern was this one site in Wyoming where there is no atrazine use. To me it appeared to be quite a pristine place, it was actually a lovely place to camp, but also had the highest percentage -- highest proportion of hermaphrodites out of all the sites.

Here is what -- just an example of one of the animals. If you do the sectioning through not only testes with oocytes, but some of the testes actually have what appear to be ovarian vesicles resident in them as well.

So, what is going on? Let's sort of below that up a little bit. The North Plat River flows this way. Maybe everybody knew this. I didn't know it. The North Plat River flows this way, so that it is not atrazine traveling from Nebraska, but it originates in Colorado and I'm working now with William Battaglin. We're sampling in these areas in the spring, the USGSs and in the summer a joint effort between myself, my laboratory and the USGS. We will be exploring this source of the atrazine contamination there.

Can we blame atrazine? I mean, at this point, we're going to talk about the doses but all I can tell you is that at every site where you find atrazine above .1 part per billion, we find hermaphrodites in some proportion.

Here are those data, so we look at the hermaphroditism or the testicular oogenesis. They are probably not appropriately called hermaphrodites, because they don't have ovarian tissue. Animals with testicular oogenesis, we also had one site with animals that had predominantly testicular or gonadal dysgenesis. I will talk to you a lot about that site. This is actually -- we found out quite a bit about these animals recently. These are the atrazine levels associated with those sites. So, there is not -- I think like Dr. Ashby said, it almost looks like there is inverse correlation. We'll talk about those difficulties in a minute.

One thing that is interesting -- so now what you are looking at are the range of rana pipiens again, overlaid on the map of atrazine use. There is the route we took with all the sites. I can't see it from back here. I hope you can. There are a couple other things to consider. What I'm putting up now are ranges for other leopard frogs. It used to be sometime ago that rana pipiens was this huge -- the species with this huge range all across the United States. It was determined there were actually multiple species of leopard frogs, southern leopard frogs, northern leopard frogs, etcetera.

What is interesting is that now, instead of following just following Iadi (ph) we have formulated some other hypothesize and

we have funding now to follow drainages and river systems. What you are looking at now are some predictions of where we would find high atrazine contamination and again expect to find hermaphroditism associated with some of these sites and are you also looking at rivers now that we predict -- based on where they, are based on atrazine sales, we predict to have low atrazine contamination and we would predict low incidences of hermaphroditism. In other words, the route we took even, with the hypothesis that we had based on use, it was difficult to find an atrazine free site.

I don't think that's a weakness in the study. I think that says something about how widespread the problem could be. If you don't just find atrazine on the Cornfields -- I will show you in a minute -- it moves around quite a bit -- we have now permission to get into some of these head waters and some isolated lakes, if you helicopter -- from a guy who owns quite a bit of the water up there. So, that's one of the things we are doing now.

What I want to point out now -- it's quite interesting. These aren't my data, although I have manipulated the figure a little bit. A few months ago I met a woman named Rita Kenadia (Ph) who was finishing her Master's thesis and she -- working at SS State. She came to see me because what she was doing was -- let me go back. What

she was doing was she was working along these contact zones, using mitochondrial and nuclear DNA analysis. She was trying to determine if these were real boundaries. So, for example, this yellow should be rana blaireye (ph) and all this pink should be rana pipiens.

It turns out that coincidentally, this site that had the high gonadal dysgenesis, the animals that were unlike all the other rana pipiens -- one of the things she came to tell me was they are not rana pipiens, they are rana blaireye.

So, rana blaireye, which should be down here is now appearing at our site. I can't even tell if I'm pointing at the right thing from here -- appearing at our site in there in Nebraska.

So, there has been a range extension. What is more disturbing is these black circles up here, this should be the range of rana pipiens. This should be rana blaireye. What those gray circles indicate are animals that have random blaireye, mitochondrial DNA as well as rana blaireye, nuclear markers that she was looking at.

Now, we have done some work now, on this gonadal dysgenesis thing, this poorly developed gonad and we're actually formulating hypothesis now that it's actually a mechanism of resistance. We don't get hermaphroditism when we expose certain frogs. For example, certain sites in Utah, certain sites in Nebraska -- we don't get any

hermaphroditism at all. This is work subsequent to our EHP paper. I will show you what the animals look like. It appears that the animals which have a slow gonadal development, sort of metamorphosing with poor gonadal development, undifferentiated gonads, are resistant in part because they are metamorphosing after they leave the water -- I'm sorry, gonads are developing after metamorphosis or after they leave the water.

In one such population is the one down here from Nebraska, which shows the high gonadal dysgenesis. Let me just stop for a minute. Is that making sense at all or am I rambling?

So, we have animals like rana blaireye, their gonadal development seems to be delayed -- not seems to be. 100 percent of the animals seem to be delayed, either undifferentiated or small gonads, the kind of thing we called gonadal dysgenesis. They don't show hermaphroditism in response to atrazine. The hypothesis we're working on now is that they are resistant, because the gonads are differentiating. Essentially, the critical period has been shifted until after metamorphosis.

Now, the disturbing part about this figure is, everything that she has measured in here, in Nebraska, all the way into South Dakota, the mitochondrial DNA is rana pipiens. So, these are all rana pipiens.

Pipiens. The nuclear markers that she is looking at -- and these sites with high pesticide use, are all random Blaireye.

So, in other words, rana pipiens is gone. The females are clearly all rana pipiens because the mitochondrial DNA is rana pipiens, but they appear to be choosing or maybe only having a choice of these random Blaireye males, which we have identified as a potentially resistant species to atrazine. So, it is just one of the things that we are working on now. She is now joining my lab for a PhD. to combine development in endocrinology, et cetera, with the population and genetics and kinds of things that she is doing. But it speaks to the impact. There was something about robust populations came up. One problem is, if you go to a site -- and we went to some sites where you find no frogs. It is hard to say, well, whether or not the frogs were affected by pesticides, there is no way to tell why they went away once they are gone.

At the same time, just because you find frogs there and I think we had discussion about robust populations, it doesn't mean that they are what they used to be. She is now looking, for example, at the possibility of genetic bottle necking. In this case it could also be hybrids, which may or may not be driven by pesticides. There has to be ways to get at those kinds of answers.

What we're going to do now is -- we have had these control studies where we can identify end points. We can try to identify mechanisms and probe into those mechanisms. Although control these aren't real studies. We have now gone and tried to do -- I mean, they aren't real world, I should say. We have now gone into the real world and tried to do a study where we looked at whether or not the effect occurred and whether we -- and whether or not there was an association of contamination. But there are a lot of things that are uncontrolled in the field.

So, now what we're going to do is look at some of those uncertainties in the field. I'm going tell you how we try to make more real laboratory experiments to try to look at what some of these factors may be and to try to control some of these real factors that we might be interested in.

One problem is -- you are looking at a field. I'm going to show you this again. In the winter, just as an ice starts to melt, when the rana pipiens breed -- this is a field in Nebraska. Everything is covered in water. Even though you might try to look at plots, one where there is atrazine in use and one where there is not, the water is almost continuous at least part of the year. So, this is point .3 parts per billion. You are actually looking across an organic farm that's

across the street from the corn farm that we work in. This is in the winter time, so this is when the levels should be their lowest. If you look at rain fall in the area, it is .4 parts per billion. Again, we're concerned about .1 part per billion. That's where we're seeing effects in rana pipiens as well as in xenopus laevis.

The water in this field pond, just behind the site is at .9 parts per billion. That's the same water that's taken up and irrigated -- even though atrazine is only applied twice's year at the site. the field pond is drainage from the corn field, which is then reapplied to the field so, essentially some level of atrazine is applied throughout the year.

Again, this is run off that eventually ends up in the field pond and applied back. The other problem is even in one ditch, this is the same ditch from one day to the next you can go from 15.3 parts per billion to .6 parts per billion. This came up with some of the Syngenta Ecorisk studies where one time measurements. Even multiple measurements don't give you the full range of what animals are exposed to.

Again, those are some of the difficulties in trying to make those dose response curves we have been discussing especially with field data. I think we may have to settle -- unless we can think of ways around it, for good laboratory studies that show that the animals don't

develop this way normally in the lab unless they are exposed to one of the pesticides you are interested in and then just looking for the association or presence or absence.

I don't know any other way around that. Again, here is another example. This is the ditch I was just showing you that's running off of the farm that we're working on, it's running into a wildlife refuge. This is a protected area. The other end of it runs through a pipe and floods the organic farm.

If we wanted to set off and do plots based on use, it is almost not possible, so there are a lot of uncertainties.

There are an other problems. For example, in Nebraska, I guess, there is a law that requires you to post pesticides that you are applying to your field. So, here's a sign -- the farmer -- all the farmers have given us permission and allowed us to work on the land. I have blanked out number of his site.

What he posted here -- I guess, what is posted here inside this tube if you look in there it says, this report lists the pesticides applied to this field. You can actually go and look at everything that has been applied to the field.

The problem is -- so we did. We looked. We looked in both years that we went out. The problem is, sometimes -- for example,

here you have the compound thyfluomide (ph). It's got these -- what do they call it -- these EPA numbers and then the next year those same EPA numbers show up on this government document. It shows up with tedinfeurous (ph) and syflufeuren (ph).

In fact, the farmer said he never applied thyflumoide, so he doesn't know why it's listed there on his -- on his record.

Here is atrazine. atrazine was applied twice. Each time it is applied it has a different EPA number. I don't know if that means a different formulation or not and the farmer wasn't able to tell us that. The other part of the problem is even if you can figure out what is on this cornfield, it is often times adjacent to another field. So, there might be 10 things on the Cornfield and 10 things in the cornfield and there might be another ten things on the soy field and the frogs are being collected from here.

So, I guess, all I'm agreeing with is the point in the White Paper that if you find abnormalities in the field, how can you know that those about abnormalities were caused by atrazine when there are so many other -- in this case, at one site pesticide is used.

I would argue some of the strength is we can raise those animals in the laboratory from that very population. We can raise rana -- we have rana pipiens year round. We can raise them as

controls and know that they don't develop that way in the laboratory unless they are exposed to atrazine. Still when you get to the field, again, how can you know?

So, what we did was -- this is a list of all the herbicides used at the site. This is a list of the fungicides used at the site. This is a list of the insecticides used at the site. We had the USGS, as well as Iowa Hygienics Laboratory, PTR West was too expensive.

We had them analyze several sites, the sites that we were interested in, Utah, Wyoming and two sites in Nebraska. We had them analyzed for all of these chemicals. They had methods. I can get you those. I'm not a chemist, but I could get you the methods they used. We analyzed the samples for all of these come pounds and the idea was and the idea still -- we're still working on this, is that we test each one of these compounds.

If it's really atrazine that's causing the gonadal problems, atrazine will show up, the other compounds won't. Then we did something else too. We not only tested them singly, we tested the compounds in combination as well. We did what we call the summer and the spring mixture. It turns out, all these compounds were supposedly applied in the spring, but only metolachlorine atrazine were still there in the summer, according to analysis we had done in

two different laboratories.

We tested each compound individually. We tested all 10 compounds combined, at now several doses. We started out with one dose and then we tested metolachlorine atrazine together and we also tested bicep, which is the commercial metolachlorine atrazine mix, the two herbicides. Again, we color-coated everything.

I think you are looking at what, 30, 60, 90 cages, three replicates of all the treatments, times 30 animals per replicate. Everything was color-coated. Same endpoints -- we looked at time in metamorphosis, growth, development, size at metamorphosis, gonads and as each animal metamorphosed -- first, I want to give you a little bit about the rotation, because what you are looking at now are the tanks. Each has their own color-coated air hose, each has it's own net that is color-coated that's maintained in a plastic bag to avoid contamination. Everything is covered with a drop cloth when it's moved around. We have all our treatments and controls to analyze for contaminations as well. What you are looking at now, sort of a schematic of the different treatments and we do keep them in all in a row to avoid contamination and confusion. We also do a few other things.

What I'm showing you now is that every time we do a water

change, we rotate the tank so that no one tank is ever sitting in the same place at one time. I'm going to make tank number one white. If it was here, on Tuesday, Wednesday, Thursday, Friday -- on Friday it would end up here. Three days later it would end up here. So, we have a rotation pattern. We don't do it random. There are reasons for that if you want to talk about it. We also -- I designed where the tanks go. We put -- in this particular experiment, the reason these are white, is that we put controls on to the ends and in the middle. But they weren't always there of course, they were always being rotated. What this allowed us to do is test if there were position effects, front-to-back to test if there were effects from left-to-right.

And I organized this in such a way so that all the spring samples were blocked together and all the summer samples were blocked together. I did it rather than randomized it, because if there were a left light or a front back effect, particularly if there was a left-right effect, I could separate this into two experiments and have a control as a calibrator in the middle.

You now are just looking at time to metamorphosis and average time to metamorphosis for each of these tanks and all I'm doing is putting this here to tell you there is no difference between front and back or left and right.

In other words, no matter where these controls were -- and they were never static -- every three days they were moving. We keep maps of every time we do a tank change. What I'm telling you is that there is no position effect. So, we have controlled and tested for these things. There are also atrazine tanks where we can look for for atrazine by position effect or atrazine by position by tank, if you will.

So, we control for those things. The other thing we did was, as each animal metamorphosed and got that number that I told you about, each one of those 3000 animals were individually housed in a deli cup, so that we can monitor each individual's time and metamorphosis and size at metamorphosis. For all 3000 individuals, we know when the four limbs emerged, we know how long it took it's tail to absorb and they were still maintained in treatment water. We know -- we knew eventually what the sex was. We had data on individual animals that we followed from the time the four legs came out.

What I'm going to show you -- I'm going to break it down. We looked at the individual compounds, plus the mixtures and what I want to show you actually, it is quite interesting. No metalaxle wasn't so good for tadpoles, so we lost some data on the metalaxle.

No other compound seemed to affect metamorphosis significantly. No other single compound except atrazine had some

inhibitory effect on metamorphosis. I'll tell you about it.

What was more interesting was, if you look at controls, this is now number metamorphosing and this is day on the X-axis, here is the controls. That blue line is the average time to metamorphosis. The summer mixture is metolachlor atrazine (ph). There is a delay in metamorphosis. The spring is the ten mixtures -- ten compounds together -- there is an even greater delay in average time to metamorphosis.

What is interesting is it almost seems like -- this may spark some discussion, it almost seems like the tadpoles are somehow counting the number of chemicals they are exposed to. For example, this spring mixture of the ten compounds I showed you -- there is only .1 microgram per liter of each individual compound. So, there is only a total of one microgram per liter of pesticide. Again, it is ten things at .1.

Even if I give atrazine at 200 parts per billion, it won't produce this kind of effect. Each of those compounds at .1 part per billion won't produce and effects on metamorphosis, even atrazine at 200 parts per billion won't produce this kind of effect, but when you put all compounds together, as low as .1 part per billion -- one part per billion kills them all. You get these kind of delays in metamorphosis,

which are greater than delays that you get with the two compounds. What is more is Paula Case, who is actually here today, as well, has been looking at the thyroid glands.

If you look at the thyroid glands as controls compared to animals from these mixtures, you get large -- what appear to be enlarged thyroid glands. I don't have the quantitative data yet. We are still doing the histology. Like I said, it is thousands of animals. We're looking at thyroid gland size, volume, follicle size, colloid size, all those measures of thyroid inhibition and we're also, of course, looking at first to last animal per tank. We'll looking at trends in the thyroid gland within a tank and those things. But it appears -- I mean, certainly, there is statistically significant effect with these mixture on timed metamorphosis and it may be correlated with some type of effect on the thyroid gland that we're starting to look at.

One of the other things is, I would never have looked at the data this way, but Kelly Haston -- this is actually from a different experiment -- Kelly Haston has been analyzing some data. These are animals from Utah that she is looking at and what she has done is -- here are controls and she has looked at rank order. She just looked at the first animal to metamorphosis and plotted the days to

metamorphosis, animal number 1, 2, bla, bla, bla.

What you will notice is, it seems that they start to accelerate in the last third on the tail. At .1 part per billion atrazine, you see the same acceleration. It seems like they start to slow out, to slow down when they are at the 25 parts per billion dose.

It seems and in the other data set which we'll go back to in just a minute, it seems that the inhibition of metamorphosis is due to the last third slowing down. The first two-thirds seem to do -- to be no different from the control. Does that make sense?

There is overall inhibition of metamorphosis. I'm going to show you the same thing with the mixtures. If you look at the first third of the animals, controls compared to pesticides, they are the same. There is no difference. If you look at the middle third, they start to slow down. If you look at the last third, they really start to slow down.

I don't know if that's because you're taking animals out of tank, they're now effectively at a higher dose because there is less animals in the tank to deal with the load or if it's just the slower ones are more susceptible to whatever effect the pesticide have.

Let me stop. Does that make sense?

The reason I bring it up is because there is a consequence to

this. You are now looking at time to TR, time to complete tier resorption (ph) in days. These are controls. In this case, it is nine replicates, because we're talking about the same experiment.

This is S. metolachlor, and that is atrazine. We're only looking at the last third of the animals. The first two-thirds come out -- there is no statistical significance difference. There is an overall difference, but it is because the last third are slower. If you look at controls, S. metolachlor atrazine alone delay metamorphosis by a week, almost ten days. Either one of the compounds.

If you combine the two compounds, however, metolachlor and atrazine mixed together at the same proportion that they would be mixed in bicep or bicep itself, there is a delay of about 20 days -- or about two weeks. Sorry.

When I first presented these data to some people in EPA, they asked, it is statistically significant, but is it biologically significant? Would two weeks make a real difference.

My best answer I like was one that I found when I was actually in Belize for something, but here is a cornfield. It turns out they have a leopard frog in Belize. I was supposed to be on vacation. There is my kids. They don't have anything to do with it.

There is a runoff pool or a pool here on the side of the road. If

you look in that pool, there are leopard frog tadpoles in the pool. The very next day, they are gone. So these are just dead tadpoles. That same pool that I showed you earlier has desiccated.

There are a lot of -- there is a great deal of data now that there are amphibians -- especially amphibians that breed in temporary pools that are adapted to pond drying and accelerated metamorphosis.

There has been a great deal of study including work by people on this panel to look at some of the hormones involved in that accelerated metamorphosis and involved in that response to pond desiccation.

What is the possibility that the evolution, the adaptation to pond desiccation and the hormones involved, thyroid hormone, potentially corticoids, what is the possibility that the most important thing about pond desiccation now is an increase in the concentration of the pesticides that are in that runoff, for example, such as the metolachlor and atrazine, which seem to be the only two persistent pesticides and which combined appear to inhibit metamorphosis?

I think there is a biological significance when you consider this adaptive response.

The other significance is the following. Part of this resistance I was telling you about is this. The first 25 percent of the animals in

several of the rana pipiens populations we look at have this arrangement at metamorphosis. So remember I showed you before the gonads were clearly differentiated, they had spermatids developing, they had oocytes in the ovaries?

Populations such as these in Nebraska and also Connecticut, one population we have shows this, the testis, we believe this is a testis, still has quite a bit of -- that's not just weak testis, quite a bit of cortex -- medulla in tact as well as the cortex, so it's relatively undifferentiated, and females from some of these populations almost look like a xenopus.

There is a single oocyte here and an ovarian vesical, but you don't see oocytes the way you see in some of these Wisconsin populations, some of the gonads that I showed you earlier.

So the hypothesis we are working at now is that these animals that metamorphose, these populations that metamorphose quickly, but have delayed gonadal development relative to their somatic development, may be resistant or may escape the effect because these gonads aren't going to differentiate until they are out of the water. These animals have already metamorphosed. Does that make sense?

If you look at the animals to metamorphose, those are the ones that tend to be more sex reversed or that tend to have the oocyte

statistic of the oocytes.

I guess the point I'm getting at is the more severe hermaphroditism or the more severe gonadal abnormalities tend to be in the animals that metamorphose last.

Of all the pesticides we looked at, that list I gave you of ten, the only one that produces the effects in our laboratory so far is atrazine.

Now what I'm telling you is that -- and I'm telling you not only does atrazine produce the effects, but the effects are more severe in the slower developing animals.

And now I'm telling that you when you mix atrazine with other compounds, and in this case metolachlor, you get delayed metamorphosis in the last animals to metamorphose. When atrazine is mixed with other compounds, there is -- I don't want to use the word synergism because -- there is an enhanced effect because essentially duration of exposure has been increased.

Again, by going into the field, there is value. There is uncertainties, but those uncertainties have allowed us to design a more realistic, yet controlled experiment in the laboratory.

What you are looking at now is another consequence of -- here are controls, the summer mixture, which is just the two compounds,

metolachlor and atrazine. The spring mixture, which is the ten compounds. You are looking at body weight and snout vent length, and on the X axis, time to metamorphosis. This is going to come up a couple times.

In other words, what you are looking at is you are looking at what it really means to be a tadpole. We talked about that little pin size egg with no yolk sack in the beginning. The point of being a tadpole is to get big enough so that you can metamorphose and be an insect or a carnivore. It's a growth period.

And so the longer you take, if you look at controls, the longer you take to metamorphose, the bigger you should be. In other words, if you metamorphose quickly you have had a shorter growth period, so you come out smaller. Whereas, you know, your brother, which takes longer to metamorphose or sister which takes longer to metamorphose is going to be larger because they have had a longer growth period on average.

If you mix these two compounds together, you start to reverse that trend. If you mix ten compounds together, you start to reverse it even more.

So there are consequences of not just individual compounds and the effects on the gonads, but there are also consequences when those

compounds are given in a more realistic environment, i.e., one of these chemical mixtures.

One of the other consequences especially with the ten compound mixture, although we see this a little bit with atrazine and metolachlor, here is a control at metamorphosis, a healthy animal. This is an animal exposed to the ten compound or the spring mixture. And you can see he doesn't look happy and he can't walk straight.

Apparently, there is some immuno compromise when the animals are exposed to this mixture of ten chemicals. We saw it very low frequency with the metolachlor and atrazine.

But what is happening is this animal has a microbacterium. Apparently they all have it. It's a symbion. But they don't succumb to this inner ear infection unless they are immuno compromised. And now we're at thymus and trying to do some immuno challenges to look at, try and characterize that effect. But it is another effect, again, of the combined chemicals and something we should consider.

So we have done these controlled studies. Used that to find endpoints that we then used to do field studies. Maybe the most valuable thing about the field -- certainly one of the most valuable things is we were able to use the information we got in the field to set up some real simulations in the laboratory where we can do more

realistic exposures with atrazine and its companions.

The last thing we have done, I won't be able to give you data yet because the work is still in process, is we really brought the field home.

We collected, I'm not going to even try to say the number, but hundreds of -- does anybody remember how much? A lot of water. We collected it from several sites. Sites that we expected to be contaminated, sites that we knew had high incidence of hermaphrodism.

Temporally stored it while we were on the road just overnight. Then we transported it back to Berkeley in an 18-wheeler truck with it all maintained frozen. We had somebody freeze Wholefred (ph), a solution, back at the lab so that we have -- Wholefred is just a solution we use for rearing controls. So we have control water frozen back at the lab too.

The reason for that is to answer the question that I think all critics are going to ask. And that is what if populations just vary? What if it's really just that animals, frogs from some of these sites in Nebraska, just like their gonadal dysgenesis and testicular oocytes, that is just how they are, and animals from Utah that aren't interested in that?

What we can do is and what we have been doing is each week we take out a bucket of water, we can raise Utah animals in Utah water and expect them to be normal. And if it's really just population variation, animals transplanted from sites like in Nebraska and Wyoming, for example, will still come out intersexed even if they're in that Utah water.

Whereas if you raise them in Nebraska water, if it is just normal population variation, the Nebraska water won't affect the Utah animals.

The alternative hypothesis, of course, is it's the water and we know the chemical -- what chemical contaminants are in the water. We measure it over time to make sure it's stable. USGS has been doing that for us.

If it's the water, no matter where you come from, Nebraska and Wyoming water will induce the hermaphroditism. And that's ongoing.

We also have Wholefreders controls for everybody that we're using as well, and we'll be bringing back, helicoptering water out of Montana to do similar things with the water there.

So you know, the real question, I think is, I think both the field and the laboratory studies are important. I have listened to groups argue about the relative importance and what should be done first.

I think it was the last group that was up said or maybe it was someone on the panel said maybe you do both at the same time.

I think it is important to do both at the same time so that you can characterize what is natural for the individual populations, characterize what is happening in the field, and try to simulate and characterize what they are exposed to as well as maybe bringing water back and doing the kinds of things where you can really have -- as close to as you can get as having a field in the lab where you can control everything and really determine if it's really the water that makes things like this and which compounds in the water.

So that's the sort of lab field, lab model that we have been working on for the last three years. I don't know if I should pause here for questions. Because the next thing I want to do is really address this cause and effect question. Some of the questions that the panel is charged with about dose response, et cetera and mechanism.

DR. ROBERTS: Let's go ahead and ask the panel if they have any questions on anything you have presented so far.

DR. HAYES: Yes, please.

DR. ROBERTS: Dr. Skelly?

DR. SKELLY: I have some questions about your field methods which pertain to the Nature and EHP papers. I guess they are

important because you are continuing to use these field sites.

DR. HAYES: Yes.

DR. SKELLY: I guess I'm curious how you selected those eight locations? You talked about it briefly, but I would be interested in some more details. And then for those general locations how you selected specific wetlands.

And then how you -- you said you collected 100 animals. I think Dr. Ashby was referring to -- the next couple sentences in there said you analyzed 20 of them. Maybe you can clarify that. I think that's what he was referring to.

But how did you actually collect those animals? And specifically, you said you were targeting metamorphs and you were basing that on size. Were you identifying the metamorphs before you picked them up or were you doing that afterwards? And also -- well, that's enough for now. Why don't you chew on that a little.

DR. HAYES: Let me back up for a second. It might be useful to have this out.

The first part of the question is how did we pick the sites. One is I looked at this map, and I made some predictions about what is going to be a reference or what we call a control site. What is going to be a reference site. What is going to be a contaminated site.

I based my hypotheses on what was reference and what was contaminated based on this map which was on sales.

DR. SKELLY: Did you sit there in Berkeley before you got on the road and just picked counties and picked like eight counties and a set of back-ups?

DR. HAYES: I picked the site in Utah in part because I was working with somebody in Utah Fish and Wildlife who knew where there were healthy rana populations. And those were animals that we already had in a colony in a laboratory.

And then because I was using one in Utah I wanted a nearby site that would likely be contaminated. So I went to this county which, I believe, is Cash County, because there is golf courses and cherry growing there. So I wanted sort of paired sites.

I picked everything along a parallel. I mean, I'm making a joke about I 80, but I picked it on a parallel because of the differences in the development times as you go north south.

DR. SKELLY: (Inaudible) You got an altitude; no gradient there, though, too. Right?

DR. HAYES: Yes. We have all that in the paper and the GPS. Yes, you are correct.

But we recorded that and took -- originally, we were going to

collect in Nevada, for example. There are just no viable populations there and the state wasn't going to give us a permit to collect 100 from any site.

We did get some animals -- there was die off here or something, but we haven't analyzed them and, of course, there was a die off on Native American land. Somebody was working there, but we didn't use any of that. So the site from Utah was where we knew healthy populations, then we chose a site -- sorry it's hard see from way back here, we chose a site where there might be contamination.

Wyoming we picked as a reference site because we thought there would be no use in Wyoming. That that would be a good site for a reference.

In Nebraska I chose Cherry County because it was a reference site surrounded by atrazine. I knew I could find -- paired as best you can find contaminated samples or contaminated sites in Nebraska. Then I wanted several sites in Iowa, Illinois, and Indiana.

In Iowa I worked with somebody from the university who got us permission to go on the farm lands, because we wanted some runoff. He got us permission. Fred Jasen is his name. I think he is acknowledged in the paper -- got us permission to go into the wildlife

refuge.

So we had within a contaminated state, if you will, we had cornfields where we expected it to be high and then we had protected areas where they weren't using atrazine on that site because it's a wildlife refuge.

Then in Illinois and Indiana, we were given permits, but we never found a site we were comfortable enough that there were enough frogs that we felt like we could take 100 and be -- not ethical, but we just didn't feel like there was enough animals that we could do it.

The sites, in general, the counties were chosen a priori, because, in fact, when you write for the collecting permit you have to list exactly what counties you are going to. They were chosen a priori the sites. My students can tell you, we drive around until we find frogs.

So that specific sites in places we had no knowledge,like in Iowa where somebody helped us, we just drove around and went recon until we found frogs.

DR. SKELLY: So you didn't use like National Wetland Inventory maps, or anything like that?

DR. HAYES: No. I did not. No.

DR. SKELLY: And you didn't put, say, all the counties along

I-80 into a hopper, stratify them by atrazine and then select?

DR. HAYES: Like in Wyoming, we drove until we saw water. We would get out and walk and look for frogs. It's the same way I hunt in Lake Victoria in Africa.

In terms of picking the metamorphs, at each site with the exception of Cherry County, at each site we found a sample of animals which we have preserved as a reference that still had tailbud. So we knew they were newly metamorphosed. We used that snout vent length as our basis for choosing who was a new metamorph and not.

DR. SKELLY: So that is going to vary among sites, though --

DR. HAYES: That varies greatly among sites. Yes. The size at metamorphosis ranges in Nebraska from the place in the cornfield in Nebraska to about the size of my thumb to -- where is a big site? Some sites they came out this big.

DR. SKELLY: You were doing this in latter July, I guess?

DR. HAYES: Yes, because I was there for my birthday. It's July 29th. So it was in July, yes.

DR. SKELLY: Did you know that you were hitting the same point in the metamorphic period for all of these different sites?

DR. HAYES: I can't -- no, I can't know that. And I know -- what I can tell -- the reason I said except for Cherry County,

Nebraska, what I can tell you is that at each site we collected a subset of animals that still had small tail remaining. You know, sort of stage, whatever, 43, 44, 45, except Cherry County where we could not find any.

And there is some indication in Cherry County that they might even overwinter and that it might be different aged animals.

DR. SKELLY: I'm sorry I asked a long question, but how did you collect them, specifically?

DR. HAYES: Depends on where we were. So on North Plat River, we walked around with hand nets and dip nets and caught them along the river.

In Cherry County we unsuccessfully dug pitfall traps, and we ended up -- in fact, by the picture, we ended up trapping them in the grass with the same net.

In the golf course we walked, we could walk along the water and they would hop up and rush on land and catch them by hand.

In other places we walked along with nets. It depended on the terrain. And the animals behave very differently if they are in a river versus a pond versus a flooded meadow.

DR. ROBERTS: Dr. Heeringa.

DR. HEERINGA: I have a question about the lab field

simulation where you actually look at the mixtures of compounds. And as I look at the control in spring, spring summer graphs there clearly at least by the number of points on these graphs is substantial mortality differential across those which -- is that the case?

DR. HAYES: Let's pull it up. For the ten mixture, yes. And -- that would be the spring mixture.

For the summer, I don't recall that there is any difference in mortality. And we suspect, in fact, that the problem in the spring mixture is the metalaxyl (ph). That if we pull the metalaxyl out of the spring mixture, we can mix the nine compounds. Because metalaxyl doesn't do so well for the animals.

DR. HEERINGA: It was metolachlor that is part of the summer mixture?

DR. HAYES: The summer mixture is atrazine plus metolachlor. And the spring mixture is all ten of those compounds, tebupirimphos, cyfluthrin, the whole thing.

DR. HEERINGA: Since you have timed data here have you looked at all at applying a survival analysis technique to that data which would account for the censoring?

If you knew when these tadpoles died, you do know that at that point?

DR. HAYES: I know exactly what happened to each individual of these 3000.

DR. HEERINGA: Have you considered or have you done that, applied a survival analysis method that would adjust -- your endpoint that you are looking at is time to metamorphosis which is the event of interest here. But there is a competing risk which is the mortality of these individuals. And if the two were confounded in that competing risk,I think it could change the interpretation just as a lot of other human survival or event studies have.

DR. HAYES: I have not done such a thing on this data set. This is an unpublished data -- do you have a copy of it? Is that what you are looking at?

DR. HEERINGA: No.

DR. HAYES: I thought you were saying you had a copy. This is an unpublished data set. This is rana pipiens. We have repeated this now with xenopus laevis looking at multiple doses and things like that, but the analysis that you are mentioning is not something I have done thus far.

DR. HEERINGA: Just a comment, it would be a very valuable thing to add to the analysis of this particular data since you have all the time dependent measures and you know the fates of these

individuals, either death or survival and survival time to metamorphosis, so I recommend it. DR. HAYES: I might certainly talk with you more about that. Because I am not familiar with the type of analysis you are talking about, to be honest. But it sounds like something we would be interested in doing.

DR. ROBERTS: Dr. Kloas and Dr. Denver.

DR. KLOAS: To the presentater, I would like to keep on with the inhibition of metamorphosis. In rana pipiens you find something of delay on metamorphosis by atrazine. In xenopus not. Do you have any explanation for that?

DR. HAYES: There are some data, not my data that I can show you, that suggest that atrazine inhibits metamorphosis in xenopus.

Rana pipiens, the true rana pipiens vary incredibly. So even some populations of rana pipiens we get delays in metamorphosis and some we don't. I can them tell you why. I think why anyway.

So for example rana pipiens from Wisconsin, I can pull the data up, I actually have it here, don't show any delay this metamorphosis. But rana pipiens from Connecticut take about two months to metamorphose. Rana pipiens from Wisconsin, same room, same temperature, same tanks take about five months.

So there is such a larger variation that I think you don't pick up

the effect. So I don't know if it is a real biological effect that it occurs in some populations and not others but just the nature of the difference in time to metamorphosis. But treated the same way, they are very different size, very different time to metamorphosis. So it depends on the population, even with rana pipiens. It doesn't surprise me that xenopus and rana pipiens might respond differently.

DR. ROBERTS: Dr. Denver?

DR. DENVER: I have just a general point of clarification. I just wonder if your data set is more robust than what is actually presented in the public literature. Because you mentioned a number of times that you have 10,000 and more observations over the course of five or six years. And --

DR. HAYES: Sorry. That's not all atrazine, though. We do --

DR. DENVER: Okay. But assuming you have a subset of that that is atrazine, are there more data than what is presented in the public literature that can be made available?

DR. HAYES: Yes. You will see more of that today.

So for example, some of the data involved a big experiment we did to look at testosterone and estrogen levels in the larvae, and we were unsuccessful. I know Dr. Kelly has measured it. I have published and measured on steroids. We were unable to measure

steroids in the developing larvae in response to atrazine in the controls or atrazine treated.

So some of the observations were that study, which hasn't been published.

Some of them were some work we have done with the antiandrogen, suprotoacetate (ph) which I will present a little of that today. Some of them were with the ongoing study, our growout study where we are still maintaining the animals and taking blood samples in the adults.

DR. DENVER: Do you recall the sample size in the testosterone measurements in the PNAS paper? I just, I couldn't find it.

DR. HAYES: Melissa, do you know? Four controls and four -- the sample size in the testosterone measurements in the PNAS paper? How many animals per treatment?

Four individually housed animals. Yes, they were individually housed. So in this case the number of animals and the number of replicates are the same per treatment.

DR. ROBERTS: Dr. Coats, Dr. Kelly, Dr. Green.

DR. COATS: I have a question about the laboratory studies. Your paper says that the doses were confirmed by outside labs. What

does -- how does that happen?

DR. HAYES: We sent blind samples. Some of them, for example, went along with our samples from the field. We sent samples from the stock that we make. We sent samples from day zero, and then we also took samples at the end of three days when we changed the water.

What went in on day one and then we changed the water every three days and what came out, we took samples from all replicates across all doses. That was done -- when I was on the Ecorisk panel, that was done by PTRL West.

In the subsequent study PTRL West also did our sampling. And then in the rana pipiens study and some of the xenopus study it was done in triplicate by Iowa Hygenics, PTRL West, and by USGS, William Battaglin.

But now we are solely working with William Battaglin and USGS, because the data are the same from all three laboratories. All except that one sample that I talked to you about.

DR. ISOM: Did they look for any metabolites over the three days that might have been --

DR. HAYES: Yes. Those are published in the EHP paper.

De-ethyl and de-amino atrazine and one other. And in the field

samples they looked for other triazines as well.

DR. COATS: Did they show any -- I didn't see anything about the metabolites. How much they were in the lab studies.

DR. HAYES: Oh, in the lab studies.

DR. COATS: Yes.

DR. HAYES: I do have those data from the lab studies as well. They are published in the EHP paper for the field studies, but they also send data on the metabolites in the lab studies.

I don't know off the top of my head what they are. Atrazine over the course of three days decreases by 30 percent. But I don't remember what the major metabolites are over the three days.

DR. COATS: Do you think the metabolites could have any part of the activity we have seen?

DR. HAYES: I would love to -- in fact, I have written to Syngenta to ask for samples of the metabolites to test. I think it is possible.

We have tested, I don't have the data, because we're still doing the study. But we have tested nine other triazines and two species. I would love to add metabolites to one of those studies, but we don't have access to them.

DR. COATS: Do you have any body burden data on the frogs

from the lab studies or the field studies?

DR. HAYES: I do not. My recollection, when I was on the Ecorisk panel, is that it wasn't done because there was literature, I think, on bull frogs, you might have to ask Allen or Ron, that there was data on bull frogs that basically showed it didn't bioaccumulate and that it was water soluble and that was negative in the tadpoles, my recollection.

And that's why we chose not to do it in the Ecorisk panel. We froze tadpoles actually for the analysis, but it was decided we wouldn't do the analysis when I was on the panel. It is not something that I tried to do independently. No.

DR. COATS: And you didn't do them in the lab studies either then?

DR. HAYES: No, oh you mean body burdens from the field? No, I didn't even consider it, and we didn't do it in the lab either.

DR. COATS: Okay.

DR. HAYES: Only the water.

DR. COATS: Okay.

DR. HAYES: And we also had food samples that went to PTRL West or the food dissolved in the water.

DR. ROBERTS: Dr. Kelly.

DR. KELLEY: This is following up on categorization of the kinds of gonads that you get with exposure to various substances. And you use the word hermaphrodite in describing the field data.

But my understanding is that your conceptual scheme for normal development of the male gonad in rana is that they start out with a distal segment that has, how can I say, female potential that may, in fact, contain oocytes. And then during maturation that part of the gonad disappears and the gonad shortens.

So when you have an animal that has frank oocytes and maintenance of that distal segment, what you are getting is failure of differentiation of the male gonad that's maintaining this more female like part. Right?

DR. HAYES: Uh-huh (Affirmative).

DR. KELLEY: So we might begin to think about a different word for describing that.

DR. HAYES: No, I agree. I should be more careful. I think hermaphrodite is inappropriate for what we find in rana.

DR. KELLEY: And what do you think about the word intersex? These words, you know, carry connotations.

DR. HAYES: Intersex, in my recollection, historically has been used interchangeably with hermaphrodite. I would be reluctant to use

the word intersex.

DR. KELLEY: Is it possible that many of the effects you see are due to developmental retardation of some sort?

So it maybe, in fact, that's part of your hypothesis for how they escape atrazine effects. So if you keep the posterior part of the gonad, it is available to plump up if it gets any estrogen and becomes vitollegenic.

DR. HAYES: Yes.

DR. KELLEY: Right. So that's possible. And you don't -- you tried, I'm confused about this. But you have tried to breed some of these animals?

From the original study you didn't have enough animals left to breed. I guess that was the xenopus study.

But from these rana animals, do we have any data on what the fertility of an animal with a maintained oocytes and good testicular tissue would be?

DR. HAYES: We have from the xenopus, yes, we have tried multiple times to breed the xenopus. Like I said -- and correct me if I'm wrong, but my experience is that the males become sexually mature earlier and at a smaller size and then the females take a little bit longer.

And we have tried to breed within that, you know, not to bring in other animals to breed with our treated animals, but often what we think are the little males, as I said earlier, eventually we will inject them and try to get them to breed and they'll lay eggs.

The problem is you can't prove that they used to be males. You just now know you have a preponderance of females, so, in xenopus.

In rana now we do have a colony that is -- we actually have atrazine treated, Nebraska water treated, Wyoming water, they have been raised in a whole bunch of different waters. And they are now adult size. And we have blood samples and we are starting to measure hormones, but we haven't attempted any breeding.

And as you know, rana is a tempered species, will be a little more difficult.

DR. KELLEY: And Witchie in his early studies of estrogen treatment, partial estrogenization of xenopus pointed out that although at early stages you had ovaries that were -- you had gonads that were -- contained both testes and ovarian tissue, which is very unusual.

At later stages it looked to him like some internal regulatory process happened and the ovarian tissue actually went away.

And I wondered if this was also true with the animals that you

had that were atrazine treated. In your older animals, do you have a lower incidence of frank ovarian tissue or does it persist?

DR. HAYES: In the case of xenopus, again, I think that they actually completely become females. But I can't prove that. So we have a preponderance of females. We don't -- well, that's based on external and on egg laying. These are animals that are still alive. So again I can't -- they may hermaphroditic when we look inside.

For the rana we have not -- we have grown those up. I don't think we have opened any of those -- I don't think we have euthinized any to look at them. So we have ones that are now -- gosh they are a year old now. I have no idea what they look like inside.

DR. KELLEY: Here is my suggestion, which comes originally from Witchie who is no longer with us, I believe. He pointed out that if you raise -- he got complete sex conversion and then back crossed the animals when we raised groups of tadpoles in estradiol.

Some of those back crossed animals when mated with males gave rise to entirely male offspring.

His interpretation of those data was that those were feminized ZZ individuals that normally would have been male.

DR. HAYES: We have done that also.

DR. KELLEY: So you have ZZs around.

DR. HAYES: Estrogen treated, yes.

DR. KELLEY: So my suggestion would be since you know that a ZZ individual will normally -- 100 percent of the time, at least from his data, go on to become male, you know in advance of the interpretation of whether it is a real female or an estrogenized female becomes much simpler.

And he suggested that as a major advantage of the system in the 50s and 60s when he was working on it. So my suggestion is that that animal is a more appropriate animal.

DR. HAYES: We have a huge ZZ colony that we have already screened and figured out who is a ZZ female and who is just a ZW -- so we have that already.

DR. KELLEY: So you have that.

DR. HAYES: As a matter of fact, those are the animals that -- my post doc who was doing the molecular work to look at Cyp 19 and all these things, those are the animals that she is using to do that work. So we have already started that.

DR. KELLEY: Thank you.

DR. ROBERTS: Dr. Green, Dr. Kloas, Dr. Skelley.

DR. GREEN: Could we look at that slide again with that rather sickly looking frog that you collected from one of the ponds that was

atrazine contaminated?

Did you actually culture microbacterium species from this frog?

DR. HAYES: I did not culture it, but John Parker, who is the veterinarian at our university did it. I misspoke. It's not an inner ear. It's microbacterium induced meningitis.

DR. GREEN: I think that's an important observation. I'm sure you are aware a couple months ago a Canadian group published a couple papers linking atrazine amongst 13 or 15 other chemicals as being associated with immunosuppression in wild caught rana pipiens and enhancing the virulence of a very common lung pathogen in rana pipiens.

I would encourage if you have these kind of specimens collected from the field to get a postmortem exam and look for granulomatous lesions of both microbacterium and count the number of parasites. Those two things together will significantly shorten the lifespan of these wild caught frogs.

This has implications beyond the effects on the gonadal development.

One other minor point, at least in the laboratory animal environment, we don't consider a marinum (ph) species to be a symbiotic species with an aquatic animal.

DR. HAYES: Sorry. I misspoke.

DR. GREEN: It is actually an opportunistic pathogen.

It is of great interest, but something I would strongly encourage you to pursue.

DR. HAYES: Sorry. I used the wrong word. By symbion, I meant that what John Parker, the veterinarian that I'm working with, has shown is that all these animals have the pathogen. But it is only showing up in -- I misspoke, regular red. Now we have to change the color code for the mixture.

These are the only ones that actually show the disease. I didn't put in my presentation, but we have a huge data site on parasite loads of the liver and the kidneys. I have it here with me, but --

DR. GREEN: I think that might be as important as the gonadal effects of herbicides and pesticides in the end.

T lymphocyte function is something that would be useful if you have blood samples on these animals when they are alive and are able to do it. I realize it is complicated in the field.

I know John Parker quite well and his work and I'm aware. And as far as I know, the microbacterium species hasn't been speciated, which takes time and complicated PCR. It needs to be validated by several laboratories.

So it will be interesting to see if this is marinum or many of the other possible species.

DR. HAYES: He has joined my laboratory for his Ph.D.

DR. GREEN: I didn't know that.

DR. ROBERTS: Dr. Kloas, then Dr. Skelly.

DR. KLOAS: I would also, Tyrone, like to come back to the PNAS paper.

First question that arises, if you are honing on the aromatase hypothesis, why didn't you measure estradiol?

I'm quite sure you did it and you tried it at least in parallel.

DR. HAYES: I have been unable to detect estradiol in the blood of adults -- or Melissa has been unable, I should say. We can detect it in females and we were unable to detect it in the larvae.

We're looking at Cyp 19 now. And we are a little better at our tritiated water assays. But we have been unable to detect it. We have tried to measure it.

DR. KLOAS: You were successful sometime -- but I know there is a lot of difficulties, but --

DR. HAYES: I've done it before too, but --

DR. KLOAS: I'm just referring to the adult samples.

DR. HAYES: Oh, in the adults?

DR. KLOAS: Your test showed up testosterone. Next question is with a radioimmunoassay you tried to detect it.

Could you discriminate between testosterone and dehydrotestosterone?

DR. HAYES: In the work that we have done, we did not do chromatography to separate testosterone DHT. We used -- I can tell you the specificity of the antibody, but of course there is some cross reactivity with DHT. I don't know it off the top of --

DR. KLOAS: So it's androgens in general probably.

DR. HAYES: Yes. That's correct.

DR. HAYES: So if you couldn't measure estradiol at the same time, which would substantiate aromatase hypothesis? I think for me, because testosterone or androgens are much more pronounced, so in estrogens in females as in males, of course. So it looks more rather like an inhibition of steroidogenesis.

DR. HAYES: I'm going to address that later. We did measure for estradiol. In fact, I think we extracted rather large volumes of plasma. We were unable to detect and have never detected estradiol in the plasma of males. We have tried.

DR. KLOAS: I'm sorry for that. It should be present --

DR. HAYES: We can find it in females.

DR. KLOAS: At least it should be measurable.

Next point, concerning --

DR. HAYES: Wait. Sorry. That's correct, right, Melissa? Yes.

DR. KLOAS: -- the abnormalities you are referring to for -- you are covering everything. I think the biggest amount of abnormalities you showed up here, which is not -- which could not be seen in this PNAS paper is that you have unpigmented ovaries.

If you put unpigmented ovaries to the females, then you would have more or less a tendency towards feminization rather than demasculinization.

What do you think about that?

DR. HAYES: I will address that. I have some more data.

DR. KLOAS: Thank you.

DR. ROBERTS: Dr. Skelly and then Dr. LeBlanc.

DR. SKELLY: Just a couple -- one quick question first. Were you meaning to suggest based on when you were talking about Belize, that leopard frogs in North America live in temporary wetlands?

DR. HAYES: Sorry. Say again.

DR. SKELLY: You were suggesting does two weeks matter. You showed the picture of your kids in Belize and everything. And

you showed the leopard frog tadpoles. Were you suggesting that leopard frogs live in temporary wetlands in North America?

DR. HAYES: In North America, we do find leopard frogs in temporary -- yes. For example, the little runoff corn ditches and things like that are not permanent standing water.

And in other sites. There are other noncorn runoff, but we find them in little pools that dry down. But I also used that as a general example for other amphibians that would be in temporary situations as well.

DR. SKELLY: The main thing I wanted to ask is have you, are you or will you be measuring fertility effects and male and female breeding behavior of lab reared and wild caught animals across atrazine gradients?

DR. HAYES: Boy, I would like to. I think for the rana pipiens we have a big enough colony of different animals that we can get animals year-round. But could we get enough animals breeding in a big enough sample size that we can assess that, I don't know.

I would like to. Like I said, we have a colony that's been either reared in some of this frozen water that we brought back or reared in atrazine or reared in atrazine plus metolachlor. They are a year old now. They are big. But whether or not they will actually take to the

laboratory and breed, I don't know.

DR. SKELLY: What about the field research program, your field research?

DR. HAYES: Will I try to assess --

DR. SKELLY: Fertility effects, breeding behavior.

DR. HAYES: For this season, our main goal is to truly identify sites that are uncontaminated and to identify sites where the incidence of, if such thing exists, the incidence of hermaphroditism are low so we can really have control or real reference sites.

Because right now the only site we have is the Utah site.

DR. ROBERTS: Dr. LeBlanc, then Dr. Gibbs.

DR. LEBLANC: I would like to revisit the field study where you look at eight different sites at various locations across the U.S.

When I first read that, the EHP paper, I couldn't for the life of me discern any relationship between atrazine levels and gonadal abnormalities.

And if I heard you correctly in your presentation, though, you made some generalization that, at four sites where atrazine levels were expected to be higher, the incidence of abnormalities were higher or something like that.

DR. HAYES: No. All I can say is where there is atrazine there

is hermaphrodites. There is no -- it is not--

DR. LEBLANC: But there was atrazine everywhere. Wasn't there?

DR. HAYES: No. There is one site where there was no atrazine. And there's one site where there's no hermaphrodites. All I can say is that's the only atrazine-free site we can find. And it happened they're the only site with no hermaphrodites -- or no testicular ovigenesis.

DR. LEBLANC: And then you did have one site where two of the analyses said there was no atrazine and one said there was very little.

DR. HAYES: That's right.

DR. LEBLANC: So there is really no atrazine there either, but because of the discrepancy, you didn't consider that one.

DR. ROBERTS: Dr. Gibbs.

DR. GIBBS: In trying to count for some of the discrepancies in the laboratory results reported by the various research groups, I have been struck by the small numbers of individuals used to found the experimental populations.

For example, in your PNAS paper basically you're working with the offspring from three pairs.

DR. HAYES: That's right.

DR. GIBBS: I'm not an amphibian toxicologist. Is that standard laboratory practice?

DR. HAYES: It is in my laboratory that we use a mixture of three animals. We count them out 5, 5, 5, 5, 5 until all the tanks are filled. And we use three per.

We also do natural breeds and track those animals so that -- in fact, I know this came up in the white paper, so that I can actually give you siblings of the animals that I used in the Syngenta study if you wanted to use those same animals.

But we typically do three or four pairs per experiment.

DR. GIBBS: My concern is that in one of the figures you have accounted for a difference in the magnitude of the results, not the effect, but as a population difference or a stock difference.

I'm just wondering how widespread those variations are due to really found our effects or -- of the individuals used might account for some of the discrepancies among the results of the different groups.

I'm curious if in your perspective that holds any water, that perspective.

DR. HAYES: I'll account for most of the discrepancies in the next part of my talk. I'm going to address when we talk of cause and

effect the source of much of the inconsistency.

Well, let me back up. Could there be "strain" differences? Yes. There could be.

Do I think that there strain differences are the primary reason that we have discrepancies between laboratory studies that we are talking about now? No. The information I have suggests that there are other things that have confounded those data and that there are also perhaps some missing data.

DR. GIBBS: Thank you.

DR. ROBERTS: Dr. Hayes, I know you are going to move into another aspect of your presentation.

In terms of planning for a break, long do you think your next presentation, which I guess is the last phase of your presentation, formal presentation will take roughly speaking?

DR. HAYES: Depends on how many times we stop for questions. Maybe an hour. I have no concept --

DR. ROBERTS: In that case, let me suggest that we take a very short break, about 10-minute break. And then move into the last phase of the presentation. Let's reconvene in 10 minutes.

(Thereupon, a brief recess was taken.)

DR. ROBERTS: Before we continue with Dr. Hayes'

presentation, I would like to take just a moment for some public recognition of somebody who has worked very hard for the last seven years behind the scenes to make meetings like this possible.

Shirley Pursuval (ph) has worked on the SAP staff and has been frankly somebody who has really helped us get these meetings together, has made sure all of our travel gets done, we get reimbursed for travel, which I can say is very important to us on the panel, and those of us on the permanent panel wanted to take just a moment to recognize her service.

She is retiring. This will be her last SAP meeting. On behalf of the panel, we wanted to thank Shirley very much for everything she has done for us over the years.

(A flower arrangement was presented.)

MS. SHIRLEY: Thank you very much. It has been a pleasure working with you all.

DR. ROBERTS: Dr. Hayes, before you start, let me just give a heads up, an announcement.

We have a number of other individuals that wanted to comment make public comments. I wanted to alert the other public commenters that it is my intention to take most, if not all, of the public comments today so that the panel can begin our deliberations

tomorrow morning.

I would like to request that you be prepared to stay as long as it takes for us to get through the public comments today.

DR. HAYES: I'll be quick.

DR. ROBERTS: That was not intended as a message to you, Dr. Hayes.

I just wanted to give everybody a heads up that we do want to try and get through the public comments today because we have a lot of things to talk about. We want to be able to start on those tomorrow morning.

With that, Dr. Hayes, please continue.

DR. HAYES: So the real question is I guess what does it mean, and we have talked about what is robust, what is not robust both in terms of robust data and whether or not populations are such.

In a recent exchange, the following statement was made, the basic tenets required -- in terms of what does it mean, the first thing we have to do, really establish, is there cause and effect to get through the data and decide do we have enough data to determine whether or not there is cause and effect.

This is -- I'm now reading from a quote, I believe it is Solomon and Carr, the basic tenets required for establishing causal

relationships between environmental factors and disease were formalized nearly 40 years ago. And I underlined the word required.

And I have another quote here, what I do not believe -- this is from someone else, is that we can usefully lay down some hard and fast rules of evidence that must be obeyed before we accept cause and effect.

None of my nine viewpoints can bring indisputable evidence for or against the cause and effect hypothesis, and none can be required as a sine qua non. That's actually Sir Bradford Hill himself saying this so-called Hill criteria, the nine Hill criteria that they can't be required.

I do realize, though, that some of the nine criteria are important. And yesterday, Dr. Vandercrack (ph) talked about and spoke about Glen Fox and the use of the Hill criteria. I've also spoken to Glen Fox.

What I'm going to do now is I'm going to put this in that kind of framework. I'm going to use the so-called Hill criteria. Dr. Vandercrack didn't go into detail. But there are sort of nine criteria. What I'm going to do is I'm going to talk briefly about each one and talk about whether or not we have met any of these criteria and the value of them.

Strength of association, and I'll go through and tell you what each one is, consistency, specificity, temporality, biological gradient, plausibility, coherence, experimentation and analogy.

These are all from Hill's 1965 address.

They are all gray now. I'm going to go through each one, explain what is required in order to meet that criteria.

And then when I think it is met, I'm going to make it black and bold. And then we'll move on to the next one.

First, is there a strong association. Like I said, I hope that I have explained well enough and now made clear how we have decided what is a gonadal abnormality.

In most cases, they are -- even if they are below 10 percent, in most cases they are morphologies and the histologies, histopathologies that we never see in controls with the exception of the three animals in one tank out of 300 where we found the unpigmented ovary and controls.

In addition, if I'm correct, and we'll go through this a little bit later, if I'm correct that these are males, that it is only the males that are being transformed in this way, then the actual percentage of males is doubled.

So if it is 10 percent of a population and it is only males, then

it's actually 20 percent of the males that are being deformed this way. And I do believe that that is the case. So we're probably underestimating the number.

Dr. Kelley and I had a discussion. We have in place an all-genetic male producing line that we're doing that kind of work with.

I think the association is made strong by the number of replicates, by our protocol for doing things double blind. Even if we only mix three pairs of animals, each time we replicate our experiments multiple times, especially now when I know at some point I will have to come here and address and defend papers the way most people --

The other thing is we have, when I say tens of thousands, this is a couple shelves double stacked deep of slides. They are all available to anybody who wants to see any of those slides.

So the sample sizes aren't small. Even when we're talking about five percent of animals had this morphology, three percent had this, we have huge sample sizes, both from our field collected data as well as in our laboratory associated data.

And I think it at least in terms of the stuff that we're doing that there is a strong association. I know in the field there are some

problems with dose effects and what we find doing. And we're doing more to extend that data set.

One of the big things that has come up at this meeting that I want to address is sort of consistency. There are seventeen studies. I haven't seen them all. And certainly there are some published studies and past studies.

I want to spend a little bit of time addressing the consistency. I think that's at the heart of this meeting.

Whether or not there have been consistent effects, I have to be frank. I was a little surprised when I read the white paper that said that weight of the evidence didn't support and that there were no other studies supporting the gonadal problems.

And in part, for example -- I mean another scientist wrote to me, Tyrone, I agree with you that the important issue is for everyone involved to come to grips with and stop minimizing the fact that independent laboratories have demonstrated an effect of atrazine on gonadal differentiation in frogs. There is no denying this.

That was an e-mail from Jim Carr who is the lead scientist on the Ecorisk panel. That really wasn't the -- in terms of consistency, that wasn't the message that I have gotten today.

What I'm going to do now is I'm going to go through those

studies, some of those studies being Carr studies, and talk about why there might appear to be inconsistencies and how we might evaluate all of those studies together, at least in my opinion.

The study that we all just got done talking about is the PNAS study where in that paper I identified a percentage of hermaphrodites. We did not try to classify them in any way. Now we have done that. That's available for anybody who wants to see it.

Speaking of which all of my data -- John Ashby said it was impossible to reproduce. All of my SOPs have been available to the Ecorisk panel. They were developed with the Ecorisk panel, with Novartis, Syngenta. The whole three-day renewal was developed with them. All of my data has been available. I have mailed all of my data to them multiple times. I have mailed them thousands of slides more than once.

So all that has been available. And I'm available if they wanted to evaluate that to try to solve those problems.

That brings me to another study, something else that was said here today that wasn't true. These are just a scan of some data sheets. It's just an image here.

If you look there, you will see names TBH, GMM, Gloria Maglena Mendoza (ph) slide was here today. These are data sheets

from work that I conducted with Ecorisk and Syngenta in 1999.

If you look down the sex column in those data, you will see question marks and i's indicating gonadal abnormalities. Although, again, we hadn't classified them and found them then, we started producing these kind of data in exactly the same design as early as 1999.

So those are two studies now, both done by me, one with Syngenta, Ecorisk funding. One without.

There is also the work by Tavera-Mendoza. The effect is different. But still, it shows an impairment of gonadal development with exposure to atrazine. The exposure was different. It was done under different conditions, which I will address. But it is still consistent with atrazine having a negative impact on gonadal development in xenopus laevis. There are three studies now looking at xenopus laevis.

The Carr, et al., study published in Environment Toxicology and Chemistry is going to take us a little bit of time.

In part, despite that in February the statement, there is no denying this, was made some of the problems in comparing the Carr and Hayes study are demonstrated here. Carr says in April 22, wrote to me, you are right. We have not repeated your work, which was

admitted by Dr. Ashby today, and I remind everyone that I talk to about this, and did mention this clearly at the EPA briefing that I attended in March.

The problem is there were a lot of differences between the studies. And these differences, some of them explain why they got what appear to be different effects. And some of them I think are quite detrimental to their study, but I'll have more to say about that later.

At one point on April 22nd, though, he also said after saying he didn't -- oops, we're back there again. Sorry about that.

What I want to do is now point out what those differences really are and what they really mean. So this is the same figure I showed you earlier with low testes hermaphrodites and unpigmented ovaries shown on it.

And these are the data that I've pulled out of Carr's study. I stacked them as a bar. It's a little bit different. The lobe testes are what he called discontinuous testes.

And probably the hermaphrodites from the pictures I have seen from his work, the hermaphrodites that are included there probably include unpigmented ovaries if you look at the photograph in the paper as well as some of what we would call hermaphrodites.

One of the points I want to make is the total percent of gonadal abnormalities at 25 parts per billion is not all that different than what we find at 25 parts per billion.

A couple things have to be addressed, however. Now you are looking at against just the Carr data. Not only do we see that there are effects at the 1, 10 and 25, but it almost looks like there is a dose response.

But there is a problem with the dosing. I think when we go through the problems with the dosing, you are going to see why some of the discrepancy arose.

I raise my animals in four liters of water, 30 animals in four liters of water from the time that they hatch until they metamorphose.

In the Carr study, somehow 60 animals were maintained in 100 mils of water initially. What I'm going to do now is do a little comparison at the so-called 25 parts per billion or 25 micrograms per liter dose.

What that means for me is that in four liters I have 100 micrograms of atrazine. So the concentration is 25 micrograms per liter. 100 micrograms and four liters.

What that means for Carr is that he is actually adding two and a half micrograms of atrazine to his animals. Meaning that I'm adding

40 times more atrazine at my 25 parts per billion dose.

When you consider that Carr has twice as many animals, my animals are actually getting 80 times as much atrazine. It is the difference between -- I have two kids. You saw that already. It is the difference between giving a spoonful of cough syrup to one of my kids or dividing it between the two.

The concentration is the same, but the dose is different. This is especially important when we consider what I just told you, that USGS and I have shown that every three days the amount of atrazine is decreasing by 30 percent.

That's probably a factor of the number of animals that the atrazine -- that are being exposed.

By the time we get out to -- I forget how many days, I think I'll show that in a minute, he has eight times less atrazine. And by the time we come to the end of this study, he has only half as much atrazine being added to his animal in terms of micrograms of atrazine per tadpole.

The concentration is the same, but the dose is different. And it is actually worse because he only changes half the water every three days. So he is actually only adding half as much as he says he is adding.

If we look at the critical stages when the gonads are differentiating, that means that Carr's dose is actually 16 times lower than mine. So even though we both looking at 25 micrograms per liter, he has a fourth of volume of water, twice as many animals and only he only adds half the water -- or changes half the water each time.

And certainly with the atrazine decreasing by 30 percent every three days, then this is significant. So his 25 -- my 25 parts per billion dose is more like a one and a half parts per billion dose, again, even though the concentration is the same.

This becomes important when we start to look at steroid hormones as well. This is data that I published back in 1995 showing that over 24 hour periods tadpoles, for example, in the case of steroids, metabolized the steroids very quickly, and two tadpoles can metabolize eight time faster than one tadpole, such that a couple micrograms of estradiol would disappear in a matter of a few hours, for example, when added the xenopus laevis tadpoles, which is probably why the estradiol didn't work in those treatments that were conducted by Carr.

So the first step is to correct the doses. If we correct the doses now, then this sort of low dose effect problem starts to go away. His

1.5 should be compared to my one part per billion dose. He has a .6 part part per billion dose which is more better compared to my .8. And then he sort of falls below .1, which is where we start to see the effect.

Then Carr did something called Cochran Armitage Test, which my understanding is is looking for a linear dose response.

For discontinuous testes, he got a P value .0003. For hermaphrodites, he got a P value of.0042.

And most biologists, I think, would accept .05. But Carr referred to P values as low as this to weak trends, Carr, et al., in the paper published in SETAC.

I asked Carr how can a P value of.0003 be a weak trend. His response was, well, it's a weak trend because when the data from the top dose are dropped, the effect is no longer significant.

So what I'm understanding now is that we're looking for a linear dose response in an experiment that has three doses. We're eliminating the top dose, the one that is really in the range that we're trying to look for. And now we have two data points which makes it even difficult to draw a line.

So the statement that they haven't repeated the effects or found significant effects I believe is a little bit faulty given the procedures

that have been used to be able to say that.

Furthermore, if you look through that SETAC paper, there are a number of things that are significant or that are considered significant at P less than .05.

These are quotes, those animals reaching NF stage 66 first in each tank were significantly larger. Estradiol treated animals, but not males were significantly longer than ethanol treated males, P less than .05. The percentage of intersex gonads also was significantly greater in the estradiol treated group, P less than .05.

So clearly other points in the SETAC paper we accept. Carr, et al., accepts P less than .05. But whenever the effects were associated with atrazine, they were referred to as weak trends.

For example, they showed increased edema with atrazine exposure, with a P value of .02, abnormal swimming with a P value of .0004, inhibition for foreleg emergence.

So there is the other reference to inhibition of metamorphosis in xenopus laevis with a P value .03, inhibition of tail reabsorption by atrazine with a P value of .04, increased discontinuous gonads, as I said, with a P value of .0003, increased intersex gonads with a P value of.0042. And originally, when the paper was submitted there was an increased laryngeal muscle size with a P value of.033, but that was

different when the paper actually came out.

There are some other issues, and right now I'm still talking about the Carr paper, but I'm moving towards the white paper because I have some concerns about this bioloading thing and the flow through thing.

And I'm going to use now some information from the Carr paper because I think we're making an error.

If we look at the first half of my animals to metamorphose, they can be any experiment, I don't remember where I pulled these data from, and the first half in the Carr experiment, they are about the same size in terms of body weight. They are approaching .6 grams.

If you look at the last half of the animals -- I just looked at the first half literally and the last half to metamorphose, they are slightly larger.

And we just had this discussion. If you take longer to metamorphose, you should be larger. Whereas if you look at the last half in the Carr study, they tend to be smaller.

So we have a huge host of adverse effects, many of them associated with atrazine, that I didn't see, the edema, abnormal swimming, inhibition to metamorphosis, as well as a growth curve that I just talked to you about as being something that I only see when

the animals are exposed to multiple pesticides or multiple stressors.

In this case, we're actually looking at controls.

Here is a bigger problem. Again, here is the first half of the animals to metamorphose in the Carr study in body weight. There is the last half.

And now what I'm plotting up for you is one you were asking -- somebody was asking me about the size of my animals. There is 3000 data points from one big study we did this year.

Again, it shows the longer the animals take to metamorphose, the larger they are. There is regression through all points. Some of these are treatments. These aren't just controls. Some of these are treatments, controls, males, females. I put everybody in there just so I can show you what the sizes should look like.

There is the average in the Carr, et al., study. Out of 3000 animals, I have a single animal that approaches the average size that Carr reported in his study.

And I think this is the condition that led the EPA in the white paper to decide that there were poor conditions and bioloading problems.

In addition to that, only 30 percent of the animals metamorphosed in 80 days. Essentially, in excess of 50 percent of the

animals were terminated. And we don't accept anything below 85 percent survivorship. Not mortality. And on average, we get 90 percent.

What the white paper presented was something that looks like this. Now, this is a different kind of figure. This is developmental stage looking at wet weight. This is metamorphic climax.

And this is really the data set we're interested in. This is the animals at stage 66.

I guess the recommendation was either four animals per tank -- but if we're trying to look at sex ratios, we cannot set up a protocol that requires to have four animals per four liters, or whatever it was.

I also think, as other people have expressed, that raising animals on a flow through is unnecessary, which I'm going to show some data suggests.

I think it will generate a lot of problems, with all due respect. xenopus don't like it. It will be an expensive set-up. It will mean that anybody who wanted to do EPA acceptable research cannot do so without industry funding or somebody who is going to pay the big bucks.

It would generate a huge water loss. It would generate a huge amount of waste, depending on what chemical you are trying to

dispose of and dealing with, a huge amount of waste. A huge cost to pay for the chemical to go through flow through.

It would eliminate the ability to move between comparative studies. Some animals simply won't live on flow through at all. And it would also -- we would be starting over from ground one.

We're talking about differences in conditions now. If we start over next year with a required flow-through system, everything we have done now will be done under different conditions and you will be throwing it out.

Everything that Vitchie and everybody else did it will be difficult to compare based on what I'm seeing here at the meeting now.

In addition, it is, again, with all due respect, it is not required.

The recommended time for healthy animals in the white paper is 55 days, metamorphic size at .63 grams. That's when you're looking at animals at stage 66.

So what they are recommending is that on flow through you can get animals at stage 66 that metamorphose by 55 days that come out on average at .63 grams.

There is 3000 data points, all raised in static renewal, every three days, the average is .67 grams in size and they metamorphose

average in 46.3 days.

So talk about weight of the evidence. There is 3000 data points.

The problem is not the bioloading and static renewal in the Carr, et al. and some of the other studies.

You're looking at a tank after three days getting ready to be renewed. That's what a tank looks like if the animals are fed properly after three days. I want you to notice two things.

One, I want you to notice how dirty the water is and imagine only changing half that water for 100 days. The other thing I want you to notice is all this stuff from someone in Michigan state. I've seen a picture of the set-up. There are no lids on the tanks. That's probably the source of the contamination in controls.

All this stuff that is on the back of our lids here. If there are no lids, it would be in the tank that it's sitting next to. Stuff grows even when you are scrubbing them and cleaning them every three days, which what we do.

Now you are looking at the color-coded net, you are looking at the plastic we lay down to prevent contamination, color-coded lid, color-coded tank. Those are scrubbed every three days. You have to take all the water out to scrub them.

The other problem that Carr, et al., experienced was the feeding. Again, you are looking at our study design on the bottom, 30 tadpoles in four liters.

Carr apparently fed his animals .4 grams every three days, if I'm getting that right from the paper.

We start off at .32. So his animals are unfed by 1.6 -- oops, 4.8 because they are only fed every three days. And then we increase the amount of food as the animals grow.

So that by the end, by days 21 to 80, our animals are getting 48 times more food than the animals in the Carr study were, which is probably why they weren't growing and the high mortality.

In addition, what disturbs me the most is -- what you are looking at now, this is a study that's looking at food levels. This was our standard food level. That's what the one X 1 was. We tried half that, 2.55 times -- you're looking at survivorship.

On this side you are looking at growth for these different food levels. That's our food level that we use in the blue. That's the food level used by Carr, Novartis, Syngeta, Ecorisk in the yellow.

Essentially, this was their food level. There is the high mortality. What disturbs me is this study was done for Syngenta. They all had all these data available. We turned in a final report. So

this feeding regime was developed as a part of the Ecorisk panel when I was a part of it. So these data were available.

I don't know why the animals were underfed by a factor of 48 or why they weren't changed. My point is please don't use that as a model for how we're going to conduct studies in the future, because the flow through is going to generate a lot more problems and it's really going to set us back, it's really going to set us back to starting all over again.

Anyway, inspite of all that, as I pointed out earlier, the gonadal abnormalities are still there. There was some statistical manipulation. But I think that this study is consistent with the other studies that have shown that even under those conditions -- I agree the conditions were poor, but I don't think you throw the study out. Even under those conditions, some of the same gonadal abnormalities were identified.

Again, here is testes and ovaries, multiple testes and ovaries identified with strong strength of association, P value .0003.

Coady, et al., was a study at Michigan state -- another thing that frustrated me yesterday was a long discussion about ethanol effects and effects in controls and oocytes in controls.

These are some data that I obtained from Michigan state on

nominal atrazine doses. There is 25 parts per billion. The 25 parts per billion nominal atrazine dose is in excess of what it is supposed to be with large pair.

What I'm mainly concerned about is that if we blow this up, there is controls, there is .1 part per billion. There is as much atrazine in the controls as there is -- in fact, there is four times .1 part per billion atrazine in the controls.

So when we're having these discussions about background hermaphroditism and how many oocytes are in the controls and is there an ethanol effect, I think data like these need to be upfront so we will know what some of the confounding effects were.

Nevertheless, I don't think -- again, I think there are problems with the study. There were no controls. But the effects that they are finding are consistent with the effects that other and better controlled studies also found.

There is the work that we did in nature. I know that we'll talk about some of the dose effects, field effects, things like that. This was the same work that we published in EHP. It was the longer version of the paper. The nature paper was published as a shorter version. We were allowed to publish more of the gonadal abnormalities so that you can actually see the full range of what we

looked at there.

There is the Reeder, et al., study. This is actually how I first learned about testicular oocytes. Here is an animal with testicular oocytes. Here is an animal with testes and ovary. This study showed gonadal abnormalities similar to what we're looking at in cricket frogs associated with atrazine exposure.

It has been dismissed because it had a P value I think of .06 or .07 or something like that.

But I think -- by the way, Hill didn't like statistics, if you go back and read. He didn't think we needed to rely on them.

But if you look at the whole body of evidence that's building, I think we can probably accept, especially if we're going to error on the side of caution, I think we can accept the .067 P value, which is what I think he got for the association.

Finally, there is a study of McKoy, et al., on the toads. One thing I want to point out -- this came out I think after the SETAC meeting in Utah. It was said about this study that lends credence to University of Berkeley endocrinologist Tyrone Hayes' hypothesis that atrazine is affecting sexual development of amphibians.

Gross, that would be Tim Gross, Dr. Gross of the Ecorisk panel, added that their findings are consistent with the previous work of both

Hayes and Texas Tech experimental toxicologist James Carr, Carr finds an effect at atrazine concentrations that are similar to what we see in the field and to what we think the toads are exposed.

I really didn't get that feeling here today that we were all -- that Texas Tech and Jim Carr and Tim Gross and everybody was in agreement that there were gonadal effects.

I guess the other thing I want to point out, and then I'm going to move on is that not only do we have nine studies show associations, but 1, 2, 3, 4 -- I don't know about the seventeen that came in now, four of those studies are Novartis, Syngenta, Ecorisk studies.

And at least two of the people involved in those studies, two of the lead authors twice have said that we were all in agreement. There is no denying this, James Carr said.

The other thing I want to point out is -- I'm not familiar with weight of evidence. But my feeling is no study is going to be perfect.

In every study, you should have measured this thing, you should have done that or you should have designed it this way.

We have nine studies, all imperfect, but we have nine studies all supporting the same endpoint, that atrazine has an effect on the gonads.

Sure. We can find a problem with my study. I can probably find

a problem with the Carr study. We can find a problem with the Gross study. But they all point towards the same thing.

I find it hard to believe that it is going to be a quintessence -- actually, I was expecting more.

Let me skip to the important part. At any rate, relative to other things that are going on with respect to new data sets that you have not seen yet, this is Jim Carr to me, I think that the past arguments over larynx, et cetera, will become trivial. There will soon be bigger issues to address. And I think that the biologists will end up on the same side on these issues. Believe me on this. Any biologist will not be able to ignore the data that will soon be coming out. This is from the leadoff in the panel.

My concern -- and I'm not accusing anybody, but my concern actually is in the other paragraph, is that, we either haven't seen anything or because there is a line here, there is a lot going on that you don't know about. Trust me on this. My differences with other panel members have to do with how the new data are interpreted. I am a biologist. Others will be using statistics to minimize the impact by the new data sets.

So my concern is that either we haven't seen everything or everything hasn't been presented. Because I thought from this I was

really expecting to see something much more robust. And now the presentation seemed to be that there is nothing robust.

At any rate, back to my other point, Hill himself said, I would myself put a good deal of weight upon similar results reached in quite different ways.

So all of this discussion about the weaknesses in the studies being that they were all done under different conditions and bla, bla, bla, Hill himself saw as a strength. If all of these studies, each with their own independent flaw, are all pointing to the same thing, doesn't that add something to the weight of the evidence?

Furthermore, Fox, who Dr. Vandercrack cited, said, in ecoepidemiology, the occurrence of an association in more than one species and species population is very strong evidence for causation.

Here, over the studies we have just looked at, we have multiple studies in the pipidae. We have several studies in ranidae. We have at least one bufonidae study and then we have Reeder's stuff on hyalodae.

It's not only that we're seeing effects under different condition, under different experimental regimes, but these effects are spread out across anurans and different families even.

I don't know how we can ignore the strength of that data as

described by Hill himself, as described by Fox himself as sort of mandated by the panel that that's how we review the data.

The specificity I'm going to kind of skip over, because even Hill so much didn't hang on this. Specificity sort of required that there be one cause, one effect.

And we all know that one chemical doesn't do one thing. I'll come back to the end of that.

I guess the only thing I want to say relative to that is all of the effects are on the gonad. They might be manifested in different ways because the gonads develop differently in the different species, but there are specific effects that seem to target males, that seem to involve demasculinization and feminization and, again, achieved in different ways.

The temporality criterion requires that the cause come before the effect.

There are several things we need to address here. In a laboratory experiment, it is really sort of a moot point. I guess it is not a moot point. The point is we don't see the abnormalities until the animals are exposed to atrazine. So the cause in this case because we're delivering it does come before the effect.

Other problems come in the field. There is two types of

temporality issues. That is one is if you are looking at animals in the field, were they exposed to atrazine when they were developing. I have already shown this. I will go through it quickly. I just want to blow up one section. We have already talked about that.

If you look at a map like this, a diagram like this, the eggs are being laid in March to April at the particular site that I chose. And then as somebody pointed out, around my birthday in late July, that's when we're going back to collect the metamorphs. At the time we left (ph) eggs at this particular site -- atrazine level's above .1 level. And at the time we went back to pick them up, they were up at 15.

So it is not likely that the atrazine disappeared. Especially, given that the larvae were growing up during this peak. It is not likely that the atrazine disappeared during the critical stages that we're concerned about.

So here I don't think there is a temporality issue. And I have already shown you that when you go back here, even before any atrazine is applied, the atrazine from last year is still measurable above .1 micrograms per liter. So the animals are likely exposed during this period.

The other temporality issue is did this these abnormalities, if they are abnormalities, occur before the advent and use of atrazine.

So for example, I'm studying these abnormalities here in 2001, '2, '3.

Reeder, et al., has gone back -- I don't think it has been published yet, but has gone back through museum specimens and shown that he gets some of these gonadal abnormalities dating all the way back to 1940.

atrazine, of course, doesn't show up until 1960. But other estrogenic compounds did show up about 1940, which in this new paper by Val Beasely (ph) he discusses the coincidence of an increase in gonadal abnormalities associated with DDT exposure, and then there is the potential of another increase associated with atrazine, with atrazine use.

One thing that comes up a lot that I disagree with is Vitchie even earlier than this talked about a I guess what he called a sex changing frog that started out hermaphroditic. It's a European ranid. Many of the papers were written on the same population. It wasn't multiple populations.

The other thing is this morphology is not one that we have been looking at or one that has been described at all. And it may very well be a natural occurring phenomena, but I don't believe it is the same phenomena that we have been looking at.

In addition, I want to point out that Hill himself said it does not -- I'm sorry. This is Rothman and Greenland, 1998, it does not follow that a reverse time order is evidence against the hypothesis that C can cause D. Rather observations in which C followed D merely show that C could not have caused D in these instances; they provide no evidence for or against the hypothesis that C can cause D in those instances in which it precedes D.

So in other words, even if you go back and find, oh, yes, there were some hermaphrodites before the use, it still doesn't rule out that atrazine is the cause now, that atrazine isn't increasing the incidence.

Here is the one that everyone wants to talk about, it seems, biological gradient.

Biological gradients suggests that there should be some kind of dose response, some kind of concentration or dose relationship between the cause and effect.

What I have heard and what I was disappointed to read, actually, in the white paper, what I disagreed with is that it is almost made a requirement if atrazine really does this thing that there has to be as is typical in toxicology a monotonic linear dose response.

If you make that a requirement, as an endocrinologist, I know can tell you you will never, I would guess, never nail any endocrine

disrupter and never get to the mechanism. I'll show you why.

Fox, again, who Dr. Vandercrack cited, says, causal relationships need not be linear or monotonic. That's just what I said, actually.

Hill in 1965 even acknowledged this and said we should then need to envisage some much more complex relationship to satisfy the cause and effect hypothesis.

I think we need to take this advice that Hill gave us in 1965.

Fox went on to say, in sum, there is a marked threshold.

And I hope I'm going to convince you that that's what we're looking at now and why that's why we're looking at.

Others are Sigmoid. He said, yes, yet others are parabolic.

I think we need to really take this to heart. Because I think that hormones don't work this way. I think I can provide you with enough examples.

Let's look again at the larynx data, data that we looked at earlier. Where there appears to be a threshold effect, whether you are looking at absolute laryngeal size at one part per billion, the slide said 10, it should have said 1, at 1 part per billion, you get threshold effect where the larynx is smaller than controls, but then it doesn't get smaller as you go out, I assume is the concern of everyone.

And even if you look at the percent above the mean, you get the same effect. At one part per billion, it levels out. Even though this is going from 80 to 90 percent, it is essentially, I would argue, a threshold effect.

Here is I think why. Here is what happens in a control female. You should recognize that. That's a gonad. That's a larynx. And in a control female, there is no testosterone. The larynx doesn't show this accelerated growth.

In a control male, testosterone stimulates the larynx to grow as shown in this schematic.

Now let's look at atrazine treated male at low doses and high doses. In an atrazine treated male, testosterone, potential, we'll talk about other mechanisms, potentially is converted to estradiol, reverses the gonad, and then there is no testosterone to make the larynx grow.

In an atrazine treated male with a high dose of testosterone, testosterone is converted to estrogen and the animal's is reversed. So the larynx doesn't grow.

It is not that atrazine is shrinking the larynx. It is preventing it from growing. Once you prevent it from growing, you can't prevent it from growing more. It is done.

Giving a higher dose of atrazine can't take away more of something that has already been taken away. I'll show you in a minute it has been taken away. We have a lot more data on hormone stuff that I think Dr. Kelley will be more pleased with as well.

So I don't know how you could expect with a higher dose, if we accept that this is a plausible mechanism, that the larynx will get smaller and smaller.

You get some dose. In this case I will argue it's one part per billion. You get some dose that diminishes testosterone. The larynx doesn't grow. Giving a bigger dose won't make it not grow more. It is already done.

Let's address this other problem now. Again, just bear with me. I'm not going to argue that it is a parabolic or an inverted U, whatever you want to call it, but we clearly did see bigger effects at the low dose than at the high dose when we looked at gonadal abnormalities in rana pipiens.

Again, let's sort of stick with the testosterone estradiol hypothesis again. And now we're going to talk about mammals for a second. I'm going to give you an analogy.

Normally, gonadotropin releasing hormone from the hypothalamus stimulates the gonadotropin's FSH and LH from the

pituitary. Now we're talking about a female. Those hormones regulate the ovarian cycle.

Estrogens in the ovarian cycle are necessary for regulation of the uterine cycle and estrogens are necessary for follicle growth and development. In other words, you need estrogen for follicle growth and development.

In fact, if you look at ovulation, estrogens increase, increase, increase. You hit some threshold effect and you ovulate.

Giving more estrogen early, giving a bigger dose of estrogen won't make you ovulate more. It won't make you ovulate faster.

In fact, if you give a big dose of estrogen, you will set the whole thing down and not ovulate at all.

You would never see a monotonic dose response in what everybody in this room understands. Throughout your menstrual cycle, estrogen levels increase, increase. They hit a peak. They hit a threshold, and you ovulate.

That threshold is different from woman to woman. It might be different from month to month, but it is not a dose response. You give a big dose of estrogen, try and make that happen earlier, you will shut the whole thing down. That's how birth control pills work.

We can envision, again, it's my hypothesis now, that we are

working on, that GNRH stimulates gonadotropins in frogs. Those gonadotropins normally in a male would stimulate testosterone. atrazine turns on aromatase. And then estrogen results in development of oocytes. Results in oogenesis.

On the other hand, if you give a bigger dose of atrazine, it is very well possible that you make enough estrogen that you shut down the pituitary and you get no oocytes.

So at this low dose, you would support oogenesis. At the high dose, you would shut it down or inhibit it potentially.

I think we have to really start thinking about what we know about endocrinology and integrating it, marrying it with toxicology in a way it will allow us to not slap on a requirement of a monotonic linear dose in order to generate cause and effect.

The other thing that is going to happen is if you look in the field -- we talked about several problems, other chemicals in the field, the levels of fluctuating up and down in the field both temporarily spatially.

Now if we accept that there are threshold effects, now we accept that there potentially could be parabolic effects -- and again, we are working on it right now, multiple populations in our lab using multiple doses of both estrogen and atrazine, but you can imagine that

this threshold effect, the percent you see of hermaphrodites might be here, for example, in terms of the dose response at one site. You might have the same threshold for another population, but a greater proportion of animals respond. You might have overlapping limits. You might have some limits that don't overlap.

So there is -- it would, if this is true, make it almost impossible with different -- sorry, varying sensitivities between populations, varying patterns of gonadal development, which we have already documented across populations, varying degrees of resistance and hybridization across populations, fluctuating atrazine levels.

I don't think you can ever expect to see if we go to a site with high atrazine and we find high hermaphrodites. I think we would be very misguided if that's what we were looking for.

But I think we need to look for the association between atrazine contamination and what we can deem as gonadal abnormalities. Get those animals from those populations back in the lab and ask did they develop that way naturally or is there something identifiable in the field and something that we can do in controlled laboratory studies.

Again, at least one member of the panel agreed. Here talking about mechanism, Jim Carr says, without this information we will not be able to determine why not all animals respond the same way, why

threshold responses differ, and if testicular oocytes observed in frogs inhabiting ag areas are due to atrazine.

So at least two of us are starting to think that way. I really want to encourage the panel -- because I honestly don't think we'll find the kinds of monotonic linear dose responses that we have been talking about here.

And Hill, of course, acknowledged, often the difficulty is to secure some satisfactory quantitative measure of the environment which will permit us to explore this dose response.

So it is going to go be difficult. Again, we have just gone through all of this. Even if you are taking sites one day to the next, you can get huge differences in atrazine levels.

If we are going to try to build a dose response curve, which one of these concentrations would we use to build that curve on.

Again, I think in a data set like this I agree. It is all over the place. All we can say is where there is atrazine there is gonadal abnormalities. Pull these populations into the laboratory and find out what we can learn there.

So I think that if we're looking for a monotonic linear response, we won't be able to make this black and bold. But I think if we use the endocrine system as a model and look carefully at the

mechanisms, then I think we can do this as well.

The other big question. Plausibility and coherence. Is there a plausible mechanism that's coherent with the types of effects that we're describing.

We have proposed one. And again, we proposed that because of what is known in mammalian cell lines, et cetera.

That atrazine turns on aromatase, that converts testosterone to estradiol and results in demasculinizing effects because of the loss of androgen and feminizing effects because of the gain of estrogen. Where is the evidence?

So again, here is our proposed mechanism you have seen before, that the inappropriate expression of estrogen causing effect on the gonads and the lack of androgen causing effect on the larynx.

Let's talk about the gonads first. One of the reasons that we believe that this is a plausible mechanism is that we have a pretty extensive data set on estrogen treatment, not just our data, but a historical data set. Controls, in controlled males, we would expect no ovaries and a normal or male type larynx.

In estrogen treated animals, we would expect them all to be female and maintain a normal female larynx.

We did a study -- actually, Roger Lou (ph) did this study

starting back when he was in undergraduate, a former student in the lab. Dr. Kelley and Brackenridge were talking actually about this exact kind of study. You are looking at animals in days.

We did a study where we treated animals with estrogen from stage 50 to stage 66. Or we treated for one week from stage 50 to stage 53 and then kept them without treatment. Or we treated them for two weeks, stage 55 to 66 was without treatment.

So everywhere you see green the animals were treated with estradiol. So they were treated for a week after hatching and then allowed to grow up, treated for two weeks after hatching then allowed to grow up or treated throughout the larval period.

This shows the different treatments from one week, two week and the full larval period.

You are now going to look at the sex ratio. This is phenotypic sex based on gonads. You are looking at the number of males and females.

Here is a control. You are going to look -- males are in blue. Females are in green.

I'm going to show you a line for 50 percent. Controls are roughly 50/50. That looks like about 40 percent female, 60 percent male.

Now I'm going to show you different treatments that we talked about. When we treat animals from stage 50 to stage 66, we get 100 percent females.

When we treat animals now from stage 50 to 55, this is that two-week treatment, we get females, we get a few males and then we get some of these abnormalities. I'll show you what that is.

And then when we treat for this one-week period, we get predominantly females, about 70 percent, some males and then we get this group of abnormalities, about 20 percent.

So this incomplete dosing in terms of the duration of dosing gives us abnormalities. Those abnormalities look like this. Discontinuous gonads or lobed testes and animals that have both testes and ovaries of varying types. Some varying types of hermaphroditism.

Somebody asked about the unpigmented ovary, however. What I'm telling you is that we can induce lobed testes and hermaphrodites with estrogen.

So those, again, I think support the hypothesis that the animals are being inappropriately exposed to estrogen. The morphologies are identical to what we see with atrazine when you give them incomplete exposure.

There is another treatment that we can give, and we actually get 33 percent unpigmented ovaries, other than atrazine and the three control animals out of 300, sporadin (ph) acetate, the anti-androgen. That's the only compound I have ever treated with when I find these unpigmented ovaries.

So what I'm telling you is a normal male presumably has some testosterone coming from his testes and he is all good. What you are looking at now, this is the unpigmented ovary. This is the mixed hermaphrodite with testes and ovaries. And this is the so-called broken testes or lobed testes or discontinuous testes.

What I'm suggesting is that atrazine, by depleting testosterone, results in the unpigmented ovary. I'm not saying that atrazine acts like sporadin acetate.

I'm saying a compound that blocks androgen action produces the same effect as atrazine which takes away -- which decreases androgen. And that estrogen exposure induces these other types of abnormalities that we have identified.

What is the evidence for effects on steroidogenesis? We did a one time measure with four animals in each treatment group. That is the data that were presented in the PNAS that we talked about today. But we have done a number of other things, a number of other studies

to address the effects of atrazine on steroidogenesis in adult males.

You will notice the fonts, et cetera, have changed. This is a presentation that was given by Melissa Lee that I'm using to present the data she has been working on for the last year.

The first thing she addressed was how to optimize conditions to best measure plasma testosterone concentrations. In this case, instead of decapitating animals, we used cardiac puncture.

We evaluated a number of things. I was unhappy, I have to say, with the field studies that were presented by Dupree (ph), by the Ecorisk panel.

Trapping the animals in traps and holding them for unspecified amount of times can affect hormone levels. We, for example, went through a lot of, as you can see here, through a lot of work to make sure that there was no association between our handling time and effects on hormone levels. There wasn't. So we addressed that.

We addressed housing effects. We asked whether or not animals should be housed singly or in groups. We did that over a number of days. The green are the group housed, and the blue are the single housed. They don't care whether they have roommates or not.

We looked at daily fluctuations to figure out what time of the day. So the gray shows nighttime measurements.

And these are just controls. These are actual, I forget, four or five animals -- Melissa? Is that about the sample size? Four or five animals, different groups of animals bled at each time. And then we bled some continuously shown in yellow to look at the effect of handling.

As I was telling Dr. Kelley, these are nighttime levels, are much higher than what we measured in the PNAS paper.

How do we characterize the effect of atrazine on hormonal profiles over time. That was just how we evaluated how to do the studies. Here is a number of other studies we did. We exposed animals to atrazine. Up to 72 days. We took blood samples at all the data shown here.

Here are some of the other differences I told you about. This is a Berkeley colony. This is a colony that we have maintained for more than 10 years. You're going to look at Berkeley control males. They do fluctuate. When we put them into the experiment, they almost always initially go down and then come back up and start cycling.

When you expose animals to atrazine, and these are all 10 parts per billion exposures, I believe, when you expose animals to atrazine, they decrease and they never go back up again.

Part of the point I want to make is that if you measure at the

wrong time during the experiment, like here for example, you will get no effect. But you are getting no effect -- in this case, you would be getting no effect because the animals aren't in season at this time, whatever that means, whereas clearly here there are significant effects.

Here is another example. Nasco animals. If you look at those levels, those are Berkeley animals. They also tend to have smaller larynges than the Nasco animals. They are not as masculine or something as the animals we order from Nasco. But even at their peak, they are at about seven nanograms per mil.

If we order animals from Nasco and acclimate them and look at control males, this is interesting, first of all they crash. They come to Berkeley. We so-call acclimate them and they crash.

If you look at the atrazine treated animals, they also crash. But this study was done before we knew to look at night. If you look at nighttime samples over the same thing, they are incredibly different at the nighttime.

Again, if you look at the wrong time during the wrong part of the year or during the wrong part of the day, you won't see the effects. But non effects aren't because the atrazine treated males are doing okay. It is because you are looking at the wrong time for the control

males.

We started, we haven't finished yet, but we started addressing another question that has come up. I think the Dr. Kloas brought it up. Maybe it is not this aromatase. Maybe it's interference with steroidogenesis some other way.

So at what point in a hormonal axis is atrazine acting. This is Melissa's diagram of the hypothalamus showing pituitary stimulating the gonad to make testosterone. And we're suggesting that that testosterone is being aromatized. But it is also possible that testosterone isn't being made, that the gonadotropin -- Dr. Cooper has certainly shown some effects on higher up in the axis.

So we did a little study. We treated animals for 27 days. What I'm going to show you is -- we took blood samples at these time points. And here is again day on the X axis. Plasma testosterone on the Y axis. Control females. We did measure females. We can't detect that atrazine does anything with females. They hardly have any testosterone.

And here now are the control and atrazine-treated males. Controls are in black. atrazine's in red.

So again, during part of the cycle, you can detect differences. During part, you can't. Then right here we took half the animals from

each group and we injected them with HCG. So we inject them with gonadotropin.

We asked, even those these atrazine treated males have diminished testosterone relative to these control males, can they respond to a pituitary challenge. We inject with HCG. There is the control males. And there is the atrazine treated males.

So the testes can respond. In fact, Melissa and I have argued over this, but it looks like they are responding in the same way in terms of the magnitude of the response before and after with controls as well as with the atrazine treated ones.

Here is the individual data that I promised to show you for the aromatase stuff. When we are getting activity, it is highly variable. We have now moved to an invitro system where we can better control things -- I mean, a total invitro exposure system, the whole thing.

We get highly variable from individual to individual, experiment to experiment. All I can tell you is we don't tend to see high or significant aromatase in controls. And we are still chasing that part of the mechanism.

We are also entertaining the possibility that there are other mechanisms acting and perhaps even more than one mechanism.

So the important things to take out of this are that you really

have to know the cycles and watch where you sample. You can cycle on one day and get an effect and on another day not get the effect. But if you don't get the effect -- by the way, this big arrow bar is due to one animal that had a huge amount of testosterone.

If you don't get the effect, it is not because the atrazine animals are recovering. It is because you are sampling at the wrong time, at least in our experience.

Also, you have to keep track of the fact that these are nocturnal animals. Day sampling, as we did for most of our stuff, is probably not the most appropriate time table to sample the animals.

Also, there may be other mechanisms working, because HCG can at least stimulate probably to the same extent an atrazine exposed male as a control. Again, there might be multiple mechanisms.

Experimentation, what Hill suggested here was that sometimes you can do an experiment. And he was mainly talking about epidemiology. If people are getting sick because they go to the well, shut the well down and see if the illness goes away.

We have talked quit a bit about experimentation. I think we have quite a bit of evidence. The only other experiment I guess would be to take atrazine away and then do field work and see if the hermaphrodites go away. That might be something to do through

temporality.

The last one I want to address is analogy. What does analogy really mean. What does analogy tell us about cause and effect.

Carr and Solomon in the learned discourses exchange also said that it was unlikely that atrazine caused these problems because, I quote, atrazine is a potent phytotoxic compound specifically designed to target a mechanism of action unique to plants, the binding of plastoquinone II during photosynthesis. As such, there is no a priori reason to suspect that atrazine would affect endocrine function in vertebrates.

So the idea seems to be that the pesticide is specific, so why would you expect it to have these kinds of effects that we're talking about.

As an analogy, I'll use DDT. It was pretty specific in what it did to insects. It inhibits mitochondrial ATP synthase, but DDT and its metabolites also inhibit prostaglandin synthesis, bind the estrogen receptor as an agonist, bind the androgen receptor as an antagonist, bind sex hormone binding globulin, induce aromatase, increase progesterone synthesis, inhibit glucocorticoid synthesis.

So here is another compound by analogy that has a pretty specific mechanism, but it does a lot of other things as well. If

atrazine does more than one thing, I don't think it should preclude us from exploring what it does to vertebrate -- in vertebrate sex differentiation.

The other analogy is there is huge literature, I think mostly out of Japan showing that there is a number of triazines pharmaceutically used that specifically inhibit aromatase. So by analogy, we have triazine.

I'm going to speak very briefly and then I'm done.

By analogy, we have triazine such as atrazine that we know at least in mammalian systems induce aromatase. We know in some rat model systems it will induce estrogen dependent or tumors associated with estrogen, estrogen exposure.

And by analogy, we have aromatase inhibitors that are being designed and tested in estrogen dependent cancer cell lines that are being designed specifically to do just the opposite.

On the one hand, we have tri-ines that we believe turn on aromatase and are associated with things like gonadal abnormalities and mammary cancer. And then we have triazines that we know inhibit aromatase but do just the opposite.

I think that analogy should help guide us as well in understanding it and understanding the mechanism.

Again, I want to point out Fox's point, in ecoepidemiology, the occurrence of an association in more than one species and species population is very strong evidence of causation.

I have pointed this out already. We have evidence, field, laboratory in the pipidae, the ranidae, the bufonidae, the hyaloidea done under all kinds of different conditions, all kinds of different exposures.

And every one -- we can argue about a .067 statistic if we want. We can argue about a lack of monotonic dose response. We can argue about all the flaws for all of those individual studies.

But when you line them all up together, every one with its flaws, whether the animals are healthy or not, they are still producing gonadal abnormalities with significant P values.

What about the mechanism? This has come up, the work of Sanderson, et al. But there has been a pretty detailed proposed mechanism. Again, GnRH stimulates the pituitary to release gonadotropins. The gonadotropins stimulate steroidogenesis through a G protein that turns on adenylate cyclase, results in a production of cyclic AMP, and, through a number of steps, turns on Cyp 19, which is the gene for aromatase, and aromatase, of course, converts testosterone to estradiol.

This whole thing is controlled over this side by phosphodiesterase. This is all proposed by John Giesy and Sanderson, et al.

And phosphodiesterase gets rid of the cyclic AMP, converting it to AMP. That's what keeps this whole system from going crazy.

Giesy and Sanderson, et al., propose that atrazine ties up the phosphodiesterase somehow. And the result is an elevation of cyclic AMP, an elevation of aromatase and increased estrogen.

So this has all been done, proposed and work done in cell lines. It has been shown in rats that estrogen increases in rats that are exposed to atrazine.

It has been also shown in rats that pituitary is down-regulated by that increase in estrogen. This is work done primarily by Syngenta. And it has also been shown that estrogen stimulates the release of prolactin.

So atrazine-fed rats tend to have high prolactin, high estrogen and low pituitary gonadotropins.

Is this mechanism that we know in some detail in mammals relevant to any of the studies that we are talking about now, relative to this issue?

In part, I promised you I would come back to it, in part, the

connection was made by Dr. Vandercrack. If we know something about the mammalian androgen receptor, it should tell us something about frogs.

Now we're going to make that point again. If we know something about the mechanism in mammals, does it tell us something about frogs.

Elevated estrogen -- we have already made the argument, i'm not going to hit you over the head again, is associated with the hermaphroditism, at least 11 studies. Again, I didn't read the seventeen that just came in.

The decrease in testosterone associated which, I think we have shown -- we have good evidence for, is associated with the laryngeal growth. These are both my studies. Industry funded and not industry funded.

The prolactin and estradiol both -- estradiol has a strong inhibitory effect on metamorphosis. And prolactin inhibits metamorphosis.

So if a mechanism like this is working in amphibians, it might explain effects in inhibited metamorphosis. I'm sorry. I forgot to put the reference in, but it is in the paper that I made available to the SAP.

It has been shown in ambystoma tigrinum that there is an inhibition of metamorphosis with a P value less than .05.

And Xenopus Laevis, Carr, et al., showed inhibition of foreleg emergence, P value of .03, inhibition of tail reabsorption, P value .04.

In rana clamitans, McKoy, et al., interestingly enough showed an inhibition of metamorphosis at 25 parts per billion, acceleration at 10 parts per billion.

And Kelly Haston in my laboratory in work that's ongoing now has shown inhibition of metamorphosis in some populations in rana pipiens and not others, which we have already talked about.

This mechanism and these changes in hormone levels that we know in some detail in mammals would explain many of the effects in amphibians.

In Sanderson, et al., John Giesy as coauthor wrote, a logical concern -- this is based on the mammalian work. A logical concern would be that exposure of wildlife and humans to atrazine herbicides, which are produced and used in large quantities, and are ubiquitous environmental contaminants, may similarly contribute to estrogen mediated toxicities and inappropriate sexual differentiation.

So this has been proposed by a member of the panel previously, the observed induction of aromatase, the rate limiting enzyme in the

conversion of androgens to estrogens, may be an underlying explanation for some of the tumor promoting properties of these herbicides in vivo.

So now we're going back again. I'm telling you just as we know things in mammals that help us out in frogs, now knowing something in frogs may be telling us something more about problems with environmental health, but more importantly, public health.

Namely, there was a study in 1990 that showed exposure to atrazine resulted in significantly increased incidence of mammary tumors, which respond to both estrogen and prolactin, and inflammation, sometimes with abscess formation of the prostate gland, which also responds to prolactin and estrogen.

And elevated prolactin and estrogen has been shown in rats.

In another rat, the sprague dawley female, Charles Eldridge wrote in 1999, nine years after the Pinter study in 1990 -- so in 1990 Pinter showed increased mammary tumors and inflammation of the prostate glands in male Fischer rats in 1990.

In 1999, Eldridge reported mammary tumors in sprague dawley rats, and wrote, the mammary tumor response is limited to one strain of one species in females.

Then again, Stevens, et al., in 1999 wrote, the carcinogenic

effect of high doses of atrazine observed in the female sprague dawley rat is a strain, sex and tissue specific response that does not have biological relevance to humans.

So my only point is we now have prostate and mammary cancer that we have known about in rats since 1990. We know that these rats have elevated estrogen and prolactin and decreased pituitary hormones.

We now have effects in amphibians that we're looking at, what, 13 years later that tend to be associated with the same hormones.

So this mechanism, again, my point being may be telling us about a lot more than frogs. I believe this meeting is about a lot more than frogs.

There is studies coming out. I have seen the abstract on induction of brain aromatase in fish in response to atrazine. Tim Gross of course with the panel in 1999, 2000 showed elevated estradiol, decreased androgens and vitollegenin in male exposed fish.

This effect is consistent with the effects in amphibians and with the hormone measurements. It is consistent with the some of the data that has been discussed, the reptile data. It is consistent with the effects in mammals, the effects in rats, elevated estrogen and prolactin and decreased androgen associated with these types of

cancers.

With regards to the relevance in humans, in one study atrazine exposure decreased intrauterine growth in Iowa communities with contaminated water. P value less than .001.

And this is just a few. atrazine exposure increases testicular cancer and prostate cancer in hispanic, R equals .41, and black, .67 farm workers, Mills 1998. Effects associated with, again, estrogen and prolactin with regards to the prostate.

atrazine exposure in drinking water increases breast cancer with a P value of less than .0001. Again, a disease associated with elevated estrogen and prolactin.

Coming up on the last side, atrazine exposure increased prostate cancer 9.4 times in a Novartis plant in Louisiana, again, an effect that has been associated with increased aromatase and prolactin.

So we have more than effects in just amphibians consistent with the proposed mechanism, fish, four major classes of amphibians and, again, data that I will suggest in a place like this in Africa where the runoff is the water that they use for cooking, I think if you told the people in that village that their water was causing some of the kinds of effects that we're debating here, then I think there would be cause

for concern because they know that water comes to their home. The same is true for us.

With regards to amphibian sensitivity, I think our canary is trying to sing. And we should listen.

DR. ROBERTS: Thank you, Dr. Hayes. Let me now open the presentation to questions by the panel.

Dr. Green.

DR. GREEN: Regarding the feeding adjustments that you made that you feel are critically important in some of your studies, did you base that on trial and error in your lab or published anuran kilocaloric requirements?

DR. HAYES: When I became involved with Syngenta -- the way we used to operate was we changed the water and renewed the solution every day, every 24 hours. We would come in at 4 a.m. change all the water.

When I initially -- I can't tell you why, but when I initially started operating with Allen Hosmeran (ph) with the panel, they didn't want such frequent renewal. You would have to address Syngenta Ecorisk to find out why.

So we did an initial study where we tried to do the change every three days, and we found high mortality, low growth and all the

animals died.

We filed a final report in 1998. We terminated the study because we created an 85 percent mortality. That was our first atrazine study that we conducted. The final report was filed with Syngenta.

Then we did a study called 98XL Food. We did two studies. 98XL Food 1 in 2. The final reports were filed for those studies.

Those studies looked at the food level that we used to feed, that was the 1 X, that we used to feed when we did the changes every day. Then we went half that because we were worried about water quality and twice that and four times.

So that it was based on what we did, but with a different frequency of water change. All of that was made available. The studies were signed off on and finalized by Syngenta and Ecorisk.

DR. GREEN: What is the food?

DR. HAYES: We feed Purina rabbit chow to everything. With the xenopus, we grind it up and dissolve it in the water. With other animals we feed it as whole pellets, and it's weighed out whether it's ground up for xenopus or whether it's thrown directly in a tank. It's a weighed set amount per number of tadpoles, as you saw.

DR. ROBERTS: Other questions?

Dr. Kelley.

DR. KELLEY: In reading over the Carr, et al., study, it is true that in their methods they say that they fed them, whatever it was, .4 grams a day. But in a later part of the paper, in the results paper they said that they actually checked the amount of food that was there. And if it was clear, they fed them more food.

So they do look like they were underfed because they were at 22 degrees and they took so long to go through metamorphosis.

But I just wanted to correct that impression. The paper does look like they did adjust the food for the animals during the course of the study.

DR. HAYES: That may have been my oversight.

DR. ROBERTS: Dr. Isom, then Dr. Matsumura, then Dr. Richards.

DR. ISOM: Perhaps I missed this, but did you measure estrogen levels in that study?

DR. HAYES: Yes. We have tried to measure estrogen levels. We have measured -- Melissa, correct me if I'm wrong, we can measure estrogen levels in females. We have never measured circulating estrogen in adult males.

DR. ISOM: You could not detect it--

DR. HAYES: We have never detected it. We could not detect it. That's right. It was below the detection limit.

Correct, Melissa?

And we have also tried measuring whole body and larvae. We have been unable to measure estradiol.

DR. ISOM: Why do you think you can't detect in the males, the ones that were treated? To me, it's logical if you are converting testosterone to estrogens and you are seeing the high levels at night, you would see the end product of that.

DR. HAYES: Not necessarily.

Again, estrogen has some role normally in the testes for sperm development, but you don't find it circulating in males.

There is local production of estrogen where it has its effect --

DR. ISOM: I would have to ask our endocrine people that. What is the half life? How long would the estrogen stay around? You see the fluctuation the diurnal or the changes in testosterone. But does estrogen stay around longer? And would it build over time or you would see higher levels of that?

I think that's important for the hypothesis to consider that.

DR. HAYES: But I don't think that elevated circulated estrogen is necessarily a requirement. Again, we're also looking at other

mechanisms that may be acting.

An increase in estrogen is consistent with the effects on the gonad, it's consistent with the oogenesis and what appear to be vitolleginic oocytes in rana pipiens. But we haven't -- all I can say is we have tried many times. We haven't been able to detect it.

DR. ISOM: What would you recommend, then, for an experimental design that we should consider to do a real complete endocrine work-up on these animals to validate this hypothesis, support this?

DR. HAYES: In our place right now, we're focusing on measuring up-regulation of Cyp 19 and doing the aromatase assays invitro.

Because it is very possible, just like in the brain of males, it is very possible that the aromatase and the conversion occurs locally and never goes into circulation.

So we're focusing right now on tissue expression of the gene for aromatase and biochemical activity.

DR. ISOM: What I tissue would you recommend to look at?

DR. HAYES: We have been doing it in the gonads. But since reading some of the stuff and John Giesy and others are doing, the brain is actually a better choice of tissue. We haven't started working

with the brain yet.

DR. ISOM: Thank you.

DR. ROBERTS: Dr. Matsumura.

DR. MATSUMURA: Have you checked specific inhibitors for those systems that you are proposing like aromatase inhibitors or PKA inhibitors? Have you done that?

DR. HAYES: Right now, we have just initiated a study that looks at atrazine in combination with miconazole, which is an aromatase inhibitor that we know to work in other frogs in our laboratory. So we are doing something like that now.

I guess your proposal is if you give atrazine plus an aromatase inhibitor, would you prevent the effect. I have two students right now who are working on that problem.

DR. MATSUMURA: I also noticed that when you added the HCG, atrazine treated ones did not recover as much as the control did.

DR. HAYES: I have argued with my colleague about this. I say the -- I guess one point is if you look at the percent response from where they started, it is the same. But the atrazine treated ones don't go up to where the controls are.

DR. MATSUMURA: What do you think?

DR. HAYES: We have only used -- that study is only done with

one dose of atrazine, and we've only done one dose of HCG.

Maybe if we give a bigger dose, maybe it will be a dose response. Maybe if we give a larger dose of atrazine, they will be able to recover. What it tells us is that the animals are still able to make testosterone. Suggests that the problem might be at the pituitary.

Did we try to measure estrogen after the HCG injection, Melissa?

I would imagine we would have. So the testes isn't ruined. It's able to respond. And the response is -- in terms of where it started and where it ended up is the same, but it doesn't go up to match the control.

DR. ROBERTS: Dr. Richards, then Dr. Coats, Dr. LeBlanc, then Dr. Denver.

DR. RICHARDS: I bring this up just because you used the data source a couple times. Most recently with respect to your arguments about temporality. It's the USGS data on atrazine concentrations.

Those data represent concentrations in the main stem Mississippi and its major tributaries.

Probably have little or nothing to do with the kinds of concentrations, exposures you would see in farm ponds or ditches in

the upper midwest.

The Mississippi, of course, is responding to hundreds or thousands and thousands of square kilometer watersheds.

The little ditches are responding to a square kilometer or less. I would expect the concentrations or the exposures would be much more episodic as you have raised in some other issues.

But it's just a poor data set, I think to try to characterize the temporality.

DR. HAYES: Initially, the way we used that was just like the atrazine used based on sales. We wanted a basis before we go out and rent two SUVs and an 18 wheeler and spend a whole month away. Are there levels that have been measured. Are there sites. And do we have reason to believe that the levels might be highest during those times.

Certainly, in the runoff, right off the cornfields where we're collecting and right off the rivers, I think that the timing is at least relevant, if not the levels, that they are applying in March, late March. They are going to be highest.

DR. ROBERTS: Did you have a follow up, Dr. Richards?

Dr. Coats.

DR. HAYES: Sorry. Remember, we're also taking

measurements before and after at the site. We're not counting on that as our measurement.

DR. COATS: Just wanted to follow up on the doses again and the measured concentrations. You didn't say measured concentrations in the PNAS paper.

And it would be very helpful if those were published, I would think. Did you have -- and you said you had decay over a three-day period.

DR. HAYES: Sorry. That wasn't from the PNAS paper. Those are brand new data that I just did with Battaglin maybe within the last six or seven months.

DR. COATS: Are those data available?

DR. HAYES: I can make those data available. Those are brand new data. Those weren't done along with the PNAS paper.

The question had come up -- put some discussions between myself and members of the Ecorisk panel about static renewal and bla, bla, bla. So we did a study where we maintained the animals or maintained buckets without tadpoles and took the measurements.

That's fairly recent data.

DR. COATS: Without tadpoles, you took the measurements?

DR. HAYES: With and without tadpoles. It is associated with

the tadpoles.

DR. COATS: And how much decay did you get over a three-day period?

DR. HAYES: It goes down by 30 percent.

DR. COATS: Was that in the .1 part per billion or 25 part per billion?

DR. HAYES: We did two doses, as I recall. It was a 25 and a .1.

DR. COATS: And it was the same percentage decay over both times?

DR. HAYES: Yes.

DR. COATS: My other question was about the summer set that you looked at, metolachlor and atrazine together. Was that at 25 parts per billion?

DR. HAYES: No. We looked at atrazine and --

I don't remember the proportions off the top of my head, but they were mixed at exactly the same proportions. They were mixed in bicep.

We've also conducted the studies where they were mixed at the proportion that we find them in the field, which is close to what you find in --

DR. COATS: At what concentration?

DR. HAYES: We looked at one at .1, and we've looked at one at 10, I believe, in initial study. And in a study we just completed, we did .11 and 10 in a xenopus laevis study.

DR. COATS: The graph you showed us about the maturation rate, body weights, which concentration was that at or was that a pooling of all?

DR. HAYES: Which?

DR. COATS: You showed the summer chemicals, atrazine metolachlor. You showed a --

DR. HAYES: That's the .1. Anything higher than that died. You're talking about --

DR. COATS: Not of the 10 mixture. Just of the atrazine and metolachlor.

DR. HAYES: That's .1.

DR. COATS: Thank you.

DR. ROBERTS: Dr. Hayes, as a short follow up to one of Dr. Coats' questions for clarification.

To what extent are your data available to EPA as they try and sort through this? If they pick up the phone and say, hey, can we look at your data on -- to what extent --

DR. HAYES: Depending on what you want, I have already sent a huge amount of data and SOPs and protocols through Tom Steeger. But anybody in this room is welcome to get raw data, transcribed data. Every animal I have talked about is available in the lab. Every slide is available in the lab. You can anything you want. It's a public university. It is all yours. Anything you want, just call and let me know.

DR. ROBERTS: Thank you.

Dr. LeBlanc and then Dr. Denver and Dr. Green.

DR. LEBLANC: I would like to revisit the issue of the hypothesis that atrazine increases aromatase activity and thus the conversion of testosterone to estradiol.

I agree with you that an increase in serum estradiol really isn't a requirement of the hypothesis that the induction could result in tissue specific induction and tissue specific increases in estradiol levels.

But with atrazine treatment, you observed a tenfold decrease in serum testosterone. So the question is where is the estradiol.

DR. HAYES: I would love to show a beautiful graph with lots of estradiol.

DR. LEBLANC: It should be up in serum. And it is not.

DR. HAYES: Like I said, we're exploring others mechanisms. The most consistent with the effects that we see, the feminizing effects and the demasculizing effects is that hypothesis, which is supported in other vertebrates. That's why we focused on it and we have focused on --

DR. LEBLANC: Perhaps what you should consider, I think you are really, is that you might be getting tissue specific increases in estradiol levels, but, in addition, you might be seeing some separate decrease in testosterone synthesis.

And I think your data with respect to evening testosterone levels supports that. In control animals, you see an increase in testosterone levels, which is in all probability due to an increase in synthesis.

With atrazine treatment, that doesn't occur, implying synthesis isn't occurring. Increased synthesis isn't occurring. It has been cut off in some manner.

DR. HAYES: We have worked with Doug Stocko (ph, the person who is doing molecular biology in my lab, has a whole host of cyp genes, not just cyp 19, but also steroidogenic acute regulatory -- the star proteins he's looking at and a number of other genes or a number of other enzymes are also being examined.

DR. LEBLANC: It may tie in with the LH hypothesis as well, that it may be that you are, as related to testosterone synthesis, you are interfering with the LH surge that might be controlling testosterone synthesis.

DR. HAYES: Certainly, the HCG injections, they're experiments that we need to repeat, but the HCG injections support that.

DR. ROBERTS: Dr. Denver.

DR. DENVER: I have two questions. I agree that the endocrine literature supports high doses of hormones actually having either a lower effect, no effect or even sometimes an opposite effect. We found that in my laboratory with certain assays.

Regardless of whether the effect of atrazine is endocrine mediated or not, your data in rana pipiens shows that the higher dose actually results in a lower incidence of gonadal abnormalities.

So would that lead you to predict, then, that, if you went to the field, that in sites with higher atrazine contamination you would have lower incidences?

DR. HAYES: I think it would depend on the population and the response of that population. As you know, rana pipiens, low doses of estradiol do nothing. A slightly higher dose of estradiol make 100

percent females. Really high dose of estradiol I think at the milligram levels make 100 percent males.

So given that the natural steroid can have that kind of variability in its effects, I don't know how it will make that prediction.

I think that's actually a strength of what we have done in the field paper, not a weakness, and said, look, you can't expect to find these kinds of relationships. Or maybe it means that there is not a correlation.

DR. DENVER: My second question has to do with the field studies. I appreciate the difficulties of interpreting field data.

And I was wondering if you've considered the possibility that there may be the other estrogenic compounds that are not necessarily pesticides, for example, PCBs that may be responsible for the variability that you see across different sites.

DR. HAYES: Absolutely.

DR. DENVER: Have you addressed the contamination from PCBs, for example, in these different sites? Or are any data --

DR. HAYES: We've had PCBs and organochlorines measured in the other studies, but not in the current studies, we have not.

We focused on what we knew was being applied there now to

try and generate -- there were other things like nitrate. For example, atrazine interacts with nitrate, and nitrate interacts with other chemicals.

Just already the size of the study was so enormous that we can't put everything in.

DR. DENVER: So there is a possibility that at least the incidence of intersex or whatever you want to call it at the different sites may be due to entirely different phenomena.

DR. HAYES: It could be -- phytoestrogens could be involved for that matter. It could be a host of a number of things.

We're limited also in what we can have analyzed chemically because of the cost. We have only analyzed for compounds that the farmers report that they used at the property.

We didn't go through and do a sweep of analyze for 100 things.

DR. DENVER: Well, as you got closer to industrial areas, did you see any -- I didn't recall the incidence. I'm wondering if you saw a higher incidence closer to industrial areas as you moved east perhaps?

DR. HAYES: No. I don't recall there was any relationship that way either.

DR. ROBERTS: Dr. Green.

DR. GREEN: Could you elaborate on the concerns you have about potential future studies that propose using flow through tanks and specifics about your concerns, the rate of water turnover, for example, and the detrimental effects it might have on tadpole development.

And then if we propose future grow-out experiments, do you think that flow through tanks might be acceptable for juveniles and adults?

The reason I ask is because these are space consuming and labor intensive experiments. As you know, the flow through systems, while expensive, give better control over water quality and are less labor intensive and they work quite well for adults.

But I need to hear your opinion specifically about what is wrong with the flow through tank for developmental stages.

DR. HAYES: I guess one of the differences -- I'm a basic scientist at a public institution who primarily count on things like National Science Foundation, which don't fund applied studies, that count -- funding is pretty difficult.

When I think about the cost of -- some of these compounds are expensive. The cost of the compound alone to be able to apply it through a flow through system would be huge.

What my university would charge me, depending on what the compound was -- chemical waste, to get rid of the volume of contaminated water that you would generate, what the flow through rack and systems that I know of that would be adequate would cost alone.

So, for example, the way we operate in our laboratory, when we order one of those mouse boxes you see, and it becomes an atrazine, it becomes red, green, yellow, whatever it becomes, it never gets used again.

Now you are talking about systems where tomorrow it is not going to be atrazine. It will be metolachlor. You're going to go buy a whole new system because your system is now contaminated with atrazine.

Those kinds of costs, unless you are funded by industry to do the work, simply wouldn't -- you know, it should shut any basic scientist out, because it would make it -- none of your work would be EPA acceptable.

For example, and there might be a lot gained by looking at -- like the assays we developed, we didn't develop for direct application. It was just in doing science and things like that.

So that's one difficulty.

The other difficulty is for xenopus, I don't think -- we can ask Dr. Kelley, but I don't think xenopus -- I don't think they do well in flow through water.

Even if xenopus does, I think you are going to have difficulty when you try to move to other species.

For example, if you remember my diagram moving from the laboratory model to the comparative studies, you are going to be really limited because now if you are coming to a species that doesn't live in streams, doesn't like flow through, now you have to change the whole conditions and the studies aren't comparable anymore.

DR. GREEN: I'm aware of a lot of facilities that are switching to flow through. And the definition of flow through is kind of nebulous right now because you can turn the water flow rate down on these systems to be less than five percent of the total volume per day, which is barely a trickle, but enough to keep the water quality stable, the frogs happy. It doesn't bother or stimulate their lateral line and get them excited.

From that aspect of it, it can be quite practical because you can stack a large number of animals in small rooms and do more experiments.

But I was under the impression -- I think your point is well

taken that tank contamination when you are doing pesticide, studies like this would be a problem in those systems. They might bind to the plastic or whatever and it would be pretty difficult to get them clean.

But for juveniles, is there a reason why you couldn't set up a very slow trickle through a flow-through system like this.

DR. HAYES: My first reaction for a grow-out study is that it would be even more difficult in terms of the cost and things. Because for a tadpole, you know it's only going to be a couple months.

There are other people who raise amphibians on the panel.

DR. GREEN: I just wanted to hear your opinion. You stated very earlier on that you would discourage the flow-through system. I wanted to hear it from you why.

DR. HAYES: I also think it is unfounded. I think it's unnecessary. I think the problems that came up with regards to the current and submitted studies were not related to flow through.

I think that -- again, I don't want to keep jumping on it, but my 3000 datapoints show that that's not the case, that you can achieve the kinds of results in terms of time to metamorphosis, 90 percent survivorship, metamorphosis in 45 days with a static renewal system.

DR. GREEN: Thank you.

DR. ROBERTS: Dr. Kelley.

DR. KELLEY: Well, I share your prejudices against the flow through because the tads hate it.

If you go into the field in South Africa, we once studied two related ponds that were on a golf course. One was above the other.

All the tads and the juvies were in the one above where there was no water flow through. All the adults were in the one below.

And either the adults had eaten all the tadpoles in the pond below since they are notoriously cannibalistic or the animals had segregated themselves out by preference for waterflow.

I think issues of water quality are important. There are things that we have to check. I agree with you about problems of cost.

I think there are more important issues than flow through, however, in the whole thing that have to be grappled with.

I return, and you and I have discussed this before, but let me raise this issue now again in public to the mechanism question. Of course it is secondary. You want to make sure you have this effect of atrazine.

If you do, you want to know how it does it. Your main hypothesis is that it's aromatase.

Now, in your animals that were treated with atrazine and had oocytes that yolked up, what are the only known -- what is known

about yolking up oocytes? What is the vehicle for doing that? Why do oocytes yolk up?

DR. HAYES: I'm not sure what you are -- they're filled with vitellogenin.

DR. KELLEY: Right. And where does vitellogenin come from?

DR. HAYES: Comes from the liver.

DR. KELLEY: Right. And the liver is not the gonad. Right? So it must have been the case that if estrogen caused the liver to secrete vitellogenin, that it was secreted at some point. Right?

DR. HAYES: Yes. But that's in rana, not in xenopus.

DR. KELLEY: Oh, no, no. It has been done in xenopus. Over and over again.

DR. HAYES: No, no, sorry. The atrazine yolking of the eggs was in rana, not in xenopus. Our work has been in xenopus. We have only just now started the bleeding (ph) the rana, which are a year old that I told you about.

DR. KELLEY: All right. But let me point out, and I will raise this again, that there are good endpoints for knowing if an animal has ever been exposed to a hormone. There are good endpoints for estrogen and there are good endpoints for androgen. And contemporaneous hormone measurements are misleading. Right?

You know at some point X you have Y. But you don't know what you had in between that might have caused the condition or a change in morphology that you are seeing.

One way to do that is to look at endpoints that are quite well established as being created by hormones. So vitellogenin synthesis is one.

If your aromatase hypothesis is right, you might expect to find an increase in vitellogenin synthesis. The harderian gland has been established by Chieffy (ph) to express male and female specific proteins that are under control of estrogen. There are androgenic endpoints.

So these can be used, and I would suggest should be used in an assay system if we're going to go forward with this kind of a study.

The other thing I want to point out is that both myself and my panel colleague to my right routinely measure low but detectable measures of estradiol in normal old male xenopus. They are much lower than female levels, but there is some detectable estradiol.

Maybe we could get together and go over the radioimmunoassays. That would probably be useful. Thank you.

DR. ROBERTS: Dr. Heeringa and then Dr. Coats.

DR. HEERINGA: Dr. Hayes, I would like to follow up on Dr.

Green's question, specifically related to the white paper recommendations on experimental methodology for any future studies.

In your lab protocols with the three tanks or three replications per treatment and I believe 30 subjects, 30 tadpoles per tank, when you analyzed results, let's focus on the laryngeal muscle diameter results, which are a continuous measure, did you do anything to sort of look at the inter-tank component of variability in that outcome?

You actually had charts, a box and stemp plots or distributional plots that showed the variability on that muscle size.

Did you decompose that to cross your three replicates?

DR. HAYES: Yes. We do treatment by replicate by sex, by individual --

DR. HEERINGA: What component of that total variance, say, within treatment is attributable to the replicate or specifically to the tank environment? Do you have any estimates on that?

DR. HAYES: If I can't multiply three times and ten and get 30, I assure you that I can't remember that. Occasionally, not in the PNAS studies, but we occasionally on different measures get tank effects. It depends on the species. For example, in bufo, in toads, it will often be that one cage will metamorphose.

I have always suspected that it was the first couple animals that metamorphose can stimulate the others, whether it's through hormones in the water or whatever. We have rarely seen that in xenopus.

I do know, for example, that there are shelf effects. Even if it's one foot apart, different shelves metamorphose at different times. Within a shelf in my laboratory, especially with the rotations, we don't see position or tank effects.

I did show one of those figures when I talked about rotations and I had animal controls in the left, right, middle, I did show the individual data. I treated those as individual experiments even, and did an inova and showed that there was no difference in time to metamorphosis or size at metamorphosis, whether you were at left, right, middle, or end.

And we do that in every experiment.

DR. HEERINGA: Thank you.

Just a comment. I think that the data that's present in your studies and also in the Ecorisk studies on these tank effects are extremely critical to setting up and designing for any future studies that would be done.

Tank effects, to the extent that they are present, could very much change assumptions about significance of some of the results.

DR. HAYES: I will also admit I'm not -- I know some statistics, but certainly if other people want to look at things in ways that I haven't, then that's available to anybody who wants any of the data.

DR. ROBERTS: Dr. Coats.

DR. COATS: Just a comment on the flow through possibilities.

I have worked with fish and daphnia. They do quite well in flow through. Daphnia, being a very small crustacean and the fish fry of fathead minnows or medaka (ph), start out very small. They are not bothered by the flow through.

So I don't think that should be a hindrance physically as far as -- water quality improvement would be significant, I think.

A different question, then. If you have an inverted or if you have a threshold which perhaps is exceeded at a high dose, is it not feasible, then, that you could maybe go downward from point one and create a dose response that would look more toxicological or --

DR. HAYES: I have seen something like that. I can't recall where, but not necessarily.

Like, for example, with a hormone's role in ovulation going down in dose just simply has no effect in part, you know, because there may be events increasing sensitivities leading up to the final

effect. But going down in dose wouldn't give --

DR. COATS: But if you are dealing with a population where different individuals would have different, ostensibly different thresholds or different sensitivities, you might --

DR. HAYES: I would say that's true in the menstrual cycle. I don't know if Dr. (inaudible) wants to comment.

DR. COATS: Just wondering.

DR. ROBERTS: Any other questions for Dr. Hayes? Dr. LeBlanc.

DR. LEBLANC: When you began your presentation hours and hours ago --

DR. HAYES: I was trying to get equal time with Novartis, Syngenta, Ecorisk.

DR. LEBLANC: -- you introduced an assay involving a species that we then didn't hear anything about. There was a strong color dimorphism as related to hormone treatment. You must have used atrazine with these animals. How did they respond?

DR. HAYES: That statement that was made that we've reported effects of atrazine on hyperoes (ph) was incorrect. I'm not sure where that came from.

We have done some stuff, we haven't reported it, but we have

exposed animals to atrazine. It doesn't -- Nigel, do you want to comment? It was your data.

DR. NORIEGA: I'm Nigel Noriega. I worked with hyperoleus (ph) doing some of the exposure studies. Atrazine was only done on a sample of three or four animals, which wasn't enough to do any statistics, because all of these color dimorphisms for the nonsteroids were calculated as a percentage.

And it was just too few animals to make any comment about.

DR. ROBERTS: Dr. Noriega, just to be clear for the record, your affiliation is University of California, Berkeley? Is that correct?

DR. NORIEGA: I am currently a post doctoral student within the EPA in the laboratory of Dr. Earl Gray (ph).

DR. ROBERTS: Thank you very much.

Any other questions for Dr. Hayes?

Seeing none, Dr. Hayes, thank you very much for coming here and presenting in detail the results of your studies and your conclusions and interpretations regarding those and for answering all of the panel's questions regarding that.

Thank you very much.

DR. HAYES: Thank you for the opportunity.

DR. ROBERTS: It is not my intent to entertain a give and take

among investigators who have different opinions about the data. But since the presentation you have just heard included some pointed comparisons with Dr. Carr's work, I thought I would offer Dr. Carr the opportunity, if he is interested, to very briefly comment or make any clarifications regarding his study or conclusions, if he wants.

DR. CARR: Thank you, Mr. Chairman. Jim Carr, Texas Tech University.

There were just a couple things I wanted to clarify regarding some comments made by Dr. Hayes. I'll be brief. It is getting late.

The first has to do with the issue of dose versus concentration and whether by putting tadpoles in a one liter beaker there was actually a depletion of the atrazine as suggested by Dr. Hayes to the point that the actual concentration -- or the doses were very small.

The important thing in this type of study is what actually gets into the animal. And that can be calculated using the bioconcentration factor. I have done that. I can prepare a short paper and give that to the SAP regarding bioconcentration factor, calculations in rana pipiens. That's the only data that we have.

But anyway, if you do the calculation using a value of six, and this is from a paper by Allran and Karasov, if you use a bioconcentration factor of six, and bioconcentration factor would be

defined as the concentration in the organism divided by the concentration in the matrix at equilibrium, and assuming that during the critical period of gonad differentiation we had a volume of two liters of exposure medium and at our highest dose 19.5 micrograms per liter, which was the actual measured concentration, and assuming a wet weight of the total organisms of about two grams, which is actually an overestimation for the animals at that stage of development, about stage 49, the depletion of atrazine from the medium would be about 0.6 percent.

So that's a relatively insignificant amount of atrazine that would be depleted making the concentration relatively stable over the course of our experiment.

And in the report, the final report submitted to the EPA, and it is available to the SAP, we have a graph illustrating atrazine concentrations over the course of our experiment. And they remain relatively constant. Although, at the highest concentration, the actual average measured value was 19.5 micrograms per liter rather than 25. It was a little bit less than nominal.

So we don't think there was depletion of atrazine from the tank. We think that they were exposed to fairly close to nominal concentration throughout the exposure.

And we think what is important is concentration, not dose. It is very difficult to estimate the actual dose that's getting into a tadpole that is swimming around in the stuff.

DR. ROBERTS: I was going to say unless you do the kind of measurements that Dr. Green had suggested earlier where you actually --

DR. CARR: Right. There are some data in fish and I think some preliminary data in xenopus that were presented at SETAC last year. But the full type of study has not been done. But those data are available in the report to the EPA.

There were also some issues about water quality comparisons related to our study suggesting that the quality of the water was poor after the 50 percent change.

Certainly, ammonia levels did increase during the course of our study. We have supplied data on both unionized as well as total ammonia levels, as well as pH, dissolved oxygen, conductivity, all those data are available to the SAP and to the EPA. And I would encourage anybody who is interested to look and reanalyze those data if they want to see if there is a relationship between some parameter and water quality.

It is difficult, in fact, to compare to the Hayes work because at

least in the PNAS paper and EHP paper, those data are not available.

There were also some implications. There some quotes suggesting implicitly that data might be withheld in some way from our study or other studies to the EPA. I just wanted to remind everybody that all of our data from all of the studies that the atrazine Ecorisk panel has performed are available to the EPA. They were made available by February 28th. They are available to all the SAP members. They are available to EPA. And they are there for you to conduct your own analyses if you would like to.

You may reach different conclusions based on that, but I would encourage you to take a look at those raw data if you feel so motivated. But I wanted to refute the implicit suggestion that there was some type of data withheld or incomplete data sets.

That's all I have.

DR. ROBERTS: Thank you, Dr. Carr.

Dr. Matsumura.

DR. MATSUMURA: Why did you drop the one point at the 25 microgram per liter? You disregard it. That's a big question. You really stand behind the data or you don't.

DR. CARR: That was another issue. Thank you for reminding me.

One of the other issues had to do with calling something a weak trend. Everybody here has a copy of the ETC paper. You can look in the paragraph where we report the P value for the correlation between intersex and discontinuous gonads, and the word weak is not even mentioned in that paragraph. Especially, in relationship to the correlations. And I think everyone here has the paper. They can look for themselves.

One of the things that was done in the analysis was to see what component of the data set was contributing to the trend. The data that is reported in the ETC paper as well as in the final report that was submitted to EPA and to the SAP contains the analysis for the whole data set.

One of the things that Dr. Sielkin did in his report which was submitted as an addendum to our report was to see if the trend continued at doses below 25 parts per billion.

In that particular analysis, the 25 part per billion dose was dropped out to see if the trend continued amongst the other concentrations. Now, given two or three data points, of course, you are not standing on very stable ground in terms of the correlation.

But the data that are in the ETC paper and in the final report have to do with the correlation for all of the data, for all of the doses.

That report should have been made available. It can be made available. That's Dr. Sielkin's independent statistical analysis of our data. And that would include all of the Cochran armitage tests, as well as all the other correlations and other analyses.

DR. ROBERTS: Are there any other just real quick clarifications for Dr. Carr?

DR. GREEN: What was the big announcement or finding that was alluded to in that quote from the e-mail that you said was --

DR. CARR: At that time and looking back, I probably should have realized that I would have regret doing something like that, but there was a huge data set that was getting ready to be submitted to the EPA. And there was a lot going on. I had 12 or 13 different studies that were being prepared. That is what I was referring to in that particular quote.

(Thereupon, the time was 5 o'clock p.m.)

DR. ROBERTS: Thank you, Dr. Carr.

We have more public comments to come. Let me suggest that we take a short break. 10 minutes or so. And then reconvene and we will continue with public comments.

(Thereupon, a brief recess was taken.)

DR. ROBERTS: I'm hoping we can get through the public

comments by 6:30. If we cannot, we will go ahead and adjourn at 6:30. So for those of you who are thinking about transportation and dinner and so forth, our tentative plan is to adjourn no later than 6:30, hopefully, having completed the majority or all of the public comments.

During the break, a question arose as to the availability of some studies.

Dr. Skelly, do you want to pose that question? I think there are some folks here in the audience who can answer the question for us.

DR. SKELLY: I guess I will initially address my question to Dr. Hayes.

Dr. Hayes, you mentioned that you did a study and submitted it to the Syngenta sponsored Ecorisk group that preceded the ones that were published in PNAS. And I wondered if you could share that with the SAP.

DR. HAYES: If I could share what?

DR. SKELLY: Share the paper.

DR. ROBERTS: Is there a report from that study that could be examined in the docket, entered into the docket?

DR. HAYES: The studies that I did, there was one 98XLATZ1. That was a study where the feeding wasn't appropriate, and we

terminated the study because of high mortality. I prepared a final report. I believe it was signed off on by Syngenta Ecorisk.

We then followed that with two food studies, 98XL Food 1 and 2, it was called. Those were submitted as final reports. And then we submitted -- I submitted another large study, 99XLAZT2, which was a study that examined gonads' growth, weight, development, time to metamorphosis, larynges, and a final report -- several final reports were submitted for that starting in 1999, I believe.

But can I provide it? I don't know exactly -- I have been given a letter by Syngenta that I can discuss whatever is necessary with the EPA involving my involvement. But I don't know if I can give you those documents directly. I don't know what the law --

DR. ROBERTS: Maybe we can ask a representative --

DR. SKELLY: I believe we just got it, a package that was on our chairs.

DR. ROBERTS: I'm not sure that that's what we were asking about.

Let me ask someone from the Ecorisk group if there are copies of those reports that could be placed in the docket?

Just to clarify, Dr. Hayes, there were a number of Ecorisk reports from studies from other members of the Ecorisk group that had

been entered into the public docket and were available for panel review prior to this meeting.

And during the discussions in this meeting, the mention was made of these other studies that you had done. And I don't think those were included among the studies that were on the docket. So we were just asking if you had them.

DR. HAYES: I have copies of them. I don't know if they are the property of Syngenta, Ecorisk. I know there were a bunch of studies in 1999 like the fish studies and things that were turned in. But I don't know the status of those.

DR. ROBERTS: Then we can ask someone from the Ecorisk group about the status of the studies.

I think Dr. Sielkin is here perhaps to answer that question?

DR. SIELKIN: This is Dr. Sielkin. I'm clarifying that the packet that is in front of you is the packet that I submitted that had to do with the analyses that were referred to on laryngeal size, which was the final report 99XLATZ.

It was the final -- it was really a draft report, but it was the last "final" report that was received. It was the one for which I reviewed the statistical analyses and a copy of that report as I saw it is provided to the panel.

DR. HAYES: What would be the difference between a draft and a final? I submitted several final reports, I don't know what happened to them, on those data.

DR. SIELKIN: This is the study final draft -- that's why I was hesitating with the word final draft. If you submit several final drafts, then I don't know what to call it. But this is the one that Mr. Noriega signed off on on 6/23/00.

DR. HAYES: No, I mean, there were several reports that -- for example, I submitted a report. And then you would say increase the sample size a little bit. Then I submitted another report. I don't know what happened -- I have copies of those, but I don't know if the registrant -- I don't know what the law or rule is about me making those available.

DR. ROBERTS: Let's inquire with the Ecorisk group some more then.

DR. BENZ: My name is Katherine Benz. And I was the quality assurance officer for the projects that are sponsored through the Ecorisk panel. I'm a consultant to the Ecorisk panel.

To answer the question of what is a final report, we did these studies in the spirit of the good laboratory practices. So for those of you that are familiar with the good laboratory practices, quality

assurance officers tend to look over your shoulder a lot.

And they are difficult to do in university settings. We spend a lot of time in training and writing protocols that we continue throughout all of the Ecorisk sponsored research, including protocol amendments and deviations.

But as part of that, we also did standard operating procedures. We did independent quality assurance inspections during the progress of the studies to ensure that the studies were being conducted.

At the end, we did a final report inspection of the raw data. In this case, as part of that, there is a sign off for the good laboratory practice statement as well as the quality assurance statement.

And we never signed off on the quality assurance statement because we never got a satisfactory response to some of the quality issues that were brought up in Dr. Sielkin's review.

So we, in fact, never finalized that report. It was submitted as a draft. And I believe that Dr. Sielkin's report was presented to Dr. Hayes at a meeting in San Francisco -- excuse me, at Berkeley with the understanding that we would -- those anomalies or errors in statistics or in the spreadsheets themselves would be addressed, corrected, changed, explained and a final report would be reissued with a satisfactory quality assurance statement.

I don't know if that answers the question.

DR. HAYES: I sent it back to you twice.

DR. BENZ: I don't know that we have ever gotten a statement to the best of my knowledge that the anomalies in Dr. Sielkin's report were addressed.

DR. HAYES: I've got a Fedex receipt.

DR. ROBERTS: That's fine. We were interested. It appears that there is some indeterminant status at least within the Ecorisk group regarding that report. But we were just curious if that was something that we could -- a report that we could obtain a copy of and enter into the public docket so that we could examine.

Dr. Kelley.

DR. KELLEY: Can we get the feeding reports too? Those sound like very useful pieces of information.

DR. HAYES: I don't know whose property those are. Of course, I have copies of both feeding studies and final reports that I submitted, but I don't know if I have the right -- I don't know whose property those are. I'm not a lawyer. I don't understand legal contracts.

But I have copies. If I get permission, I can turn all the final reports that I prepared for Syngenta over.

DR. MCFARLAND: I'm Janice McFarland with Syngenta. All of the information we had received from Dr. Hayes has been provided to EPA and also was provided to the SAP. We definitely allow him to release any data that he might think is confusing under the property of Syngenta to you.

DR. KELLEY: But we never got this report that he just described, nor did we ever get the feeding reports. And we still don't have them.

So if you could find them and provide them to us, that would be helpful.

DR. MCFARLAND: I believe that draft report was copied on the CD that was provided to the SAP. We can check on that, though, and provide a status.

DR. KELLEY: I'll check my CD. But it wasn't in the printout.

DR. ROBERTS: Thank you, Dr. Hayes.

DR. HAYES: My pleasure.

DR. ROBERTS: We next have scheduled a public comment from Mr. Scott Slaughter on behalf of the Center for Regulatory Effectiveness.

Is Mr. Slaughter here?

Just as a heads up, the next person I have scheduled is Mr. Jere

White on behalf of the Triazine Network.

MR. SLAUGHTER: Thank you very much. I'm Scott Slaughter, and I represent the center for regulatory effectiveness. It's late. I will try to be as succinct as possible.

CRE's primary interest in this proceeding is compliance with the Data Quality Act. The Data Quality Act requires among other requirements that EPA's conclusions regarding atrazine's effects on amphibian be based on tests that have been demonstrated to be reproducible and on data that is transparent. And by reproduceability, that includes interlaboratory reproducability.

The current data base flunks both those tests. No one has demonstrated that their test results regarding atrazine's effects on amphibians, if any, have been demonstrated to be reproducible by other laboratories.

To the best of our knowledge, all the tests to date have been solely one laboratory test. And no one has sent the exact test protocol to another laboratory to be replicated to see if they get the same or essentially the same results.

In regard to transparency, the data, the relevant data has not been available to all members of the public. For example, it was not available to CRE. CRE filed a Freedom of Information Act request

with EPA seeking essentially all data, all records within EPA's possession that refer or relate to the amphibian effects of atrazine, if any.

This went several months ago when it was filed. Recently, EPA responded by saying that Dr. Hayes has submitted seven data sets to EPA regarding this issue.

And Dr. Hayes, after review and consultation with EPA, had only agreed to release five of those data sets to CRE. We got the five data sets last week. One of those sets was encrypted. And I have very few virtues. And one of those virtues is not decoding encrypted computer disks. So I don't even know what's on that.

Based upon Dr. Hayes' testimony today as I understand it, he is willing to provide to anyone who wishes it and calls him all the data he has that is relevant to this issue. We will be calling you next week when you get back to Berkeley to get the missing data and all other data you have on this.

DR. HAYES: I'll be in Brazil next week.

MR. SLAUGHTER: Leave a number.

That was really it. Once again, I want to emphasize the importance of this SAP. I don't know whether you are all familiar with the new Data Quality Act and EPA's data quality guidelines.

It is late. If you want to ask me questions about it, I will be glad to bore you with citations to the Federal Register and the Code of Federal Regulations. But my guess is the answer to that question is no.

Comments to the SAP, we discussed this issue at some length. We also gave citations on the internet where you can obtain a copy of our Data Quality Act petition on the atrazine environmental risk assessment.

And on that petition, it was not only CRE, it was also the Kansas Corn Growers Association and the Triazine Network.

EPA's response to the -- the Data Quality Act allows interested persons to petition EPA or any other affected federal agencies to correct information that the agency has disseminated which the persons believe do not comply with the Data Quality Act standards.

Once again, one of those standards is reproducability for information of this type. And that means interlaboratory reproducibility and also test validation.

One of the critical requirements of test validation is that the test, lab test on an animal be able -- be demonstrated to be reproducible among different laboratories. One laboratory runs it. The other laboratory does the same test until they come up with

essentially the same result.

It was our understanding then and it is our understanding now that that test and criterion has never been satisfied for the atrazine database, especially with regard to atrazine's effects, if any, on amphibians and also on the whole aromatase induction issue.

This SAP, it may be the first one to address test validation issues under the new Data Quality Act and under EPA's new data quality guidelines. I congratulate you for having that honor. Actually, I may sympathize with you for being in that position.

But I want everyone to be aware here at EPA that you are operating on a new set of standards in terms of scientific information being disseminated by EPA.

Once again, based upon EPA's white paper, based upon what we, CRE, knew about the data base before the white paper, and based upon what we heard at this panel so far, at least one of those -- well, assuming that we get the data, the transparency test may be satisfied. But the reproducability test has not been.

And consequently, if this proceeding, if the atrazine review proceeds any longer or goes forward at EPA, then I think a first step, a critical first step in order to get out data that is both scientifically reliable and legally reliable is to make sure that you got validated

tests on atrazine's effects, if any, on amphibians and also on the whole aromatase induction issue.

Thank you very much. I'll be glad to try to answer any questions you have.

DR. ROBERTS: Any questions for Mr. Slaughter? Anyone want to discuss the Code of Federal Regulations with Mr. Slaughter?

MR. SLAUGHTER: I wouldn't recommend it at this point in time -- or any other, for that matter.

DR. ROBERTS: Thank you very much, Mr. Slaughter, for your comments.

My apologies to Mr. White. I misread the schedule. The next presenter is Dr. Angelina Dugan with Crop Life America, and then we will take Mr. White.

DR. DUGAN: Thank you.

First of all, Crop Life America thanks the EPA for the opportunity to address the SAP. As many of you know, CLA represents the manufacturers, formulators and distributors of plant science solution for agriculture and biotechnology.

We believe that the resolution of the question evaluation of potential developmental effects of atrazine is of great importance to the determination of environmental endocrine effects for various

chemical substances.

Overall, the development of robust testing methodology and interpretation of environmental endocrine cause and effects has proven to be technically quite complex.

More often than not, there has been a lack of consensus on studies, designs, interpretation of research results and reproduceability of data within and between laboratories.

All of this I might add is not much different than what the panel is discussing within the current proceedings. And I make this observation not to criticize any researcher or laboratory, but just to state the issue.

While the lack of concordance fuels lively academic debates, which I enjoy myself, the outcome may still not resolve or inform regulatory issues.

The Food Quality Protection Act and Safe Drinking Water (inaudible) stipulated that the test for estrogenic and other endocrine effects be fully validated as a means of ensuring reliability, consistency and the data quality for risk assessment purposes.

CLA urges the panel to develop the product of their deliberations and research recommendations in light of the same technical standards and regulatory necessities.

Our trade association has long supported the development of a validated scientifically sound screening and testing program to evaluate potential adverse endocrine effects in wildlife and mammals through both the Endocrine Disruption Screening and Testing Advisory Committee and, more newly, the Endocrine Disruption's Method Validation Subcommittee processes.

I personally served on the EDSTAC and have continued to work with EPA and the EDMVS technical experts to see the process through.

The EPA Office of Science Coordination and Policy is responsible for both the SAP and implementation of the endocrine screening and testing program. In CLA's opinion, there is an overlap in the activities of this SAP with many of the same technical issues and problems that the EDMVS is currently facing.

We believe that the efforts of both forums could benefit by the sharing of EDMVS information from the EPA office of Science Coordination and Policy and others who are involved in the EDM process.

I point out Professor Gerald LeBlanc who is also a member of the EDMVS and the SAP and also Dr. Les Touart (ph), who I believe is in the audience. He's responsible at EPA to develop, in particular,

the amphibian endocrine screens and perhaps amongst others.

For example, EDSTAC had recommended development of an amphibian metamorphosis assay to evaluate the frog thyroid axis as a screen for potential mammalian development effects.

Unfortunately, the protocol demonstration of this assay, which do consideration of several frog species and methodology, has proved to be problematic for a variety of technical reasons.

An alternative assay has since replaced the frog metamorphosis as a mammalian developmental assay, but its evaluation as a wildlife screen is still under current investigation by Dr. Touart. And at the recent June 5th EDMVS, it was communicated that the protocol demonstration of a frog wildlife assay is projected for December 2004.

CLA stresses that the technical difficulties and delays experienced by EPA and contract laboratories point out critical needs for not only continued research in the area of amphibian development, but also follow on safeguard, such as validation, to ensure clear information for either a regulatory decision or additional endocrine testing.

CLA also emphasizes to EPA and the panel that it's premature to draw any conclusions on the disruption of aromatase as a potential

human health and wildlife issue for atrazine or other environmental chemicals.

EDSTAC had also recommended the development of aromatase as an invitro mammalian assay to assess the ability of environmental chemicals to inhibit the enzyme. While there has been good progress in developing an aromatase screen, the assay is not yet available, since the initiation of the interlab validation phase is still several months away.

As far as I know, there have been no efforts to date to research aromatase uptake as a potential wildlife screen.

In closing, CLA supports thorough evaluation of research recommendations to determine potential environmental development effects on amphibians and mammalian species. However, we do not believe that the issue of potential developmental effects has been resolved for atrazine or potentially given the state of the science can be resolved for other environmental chemicals as well.

CLA urges the panel to uphold the laboratory and field data reproducibility and test validation as standards and conditions for implementing amphibian developmental assays for risk assessment purposes.

Thank you. I wish you well in your deliberations.

DR. ROBERTS: Thank you, Doctor. You jumped right into your comments. Can I ask you for the formality of introducing yourself for the record?

DR. DUGAN: Sorry. Angelina Dugan. I'm the director of science policy for Crop Life America.

DR. ROBERTS: Thank you. Let me check and see if the panel has any questions for you. Apparently not. Thank you very much.

Mr. White, who will be followed by Dr. Fawcett.

MR. WHITE: Mr. Chairman, we actually have four people who will be part of this presentation.

DR. ROBERTS: Thank you. If I could ask each of you to introduce yourself before you speak for the record.

MR. WHITE: Mr. Chairman, members of the committee, my name is Jere White. I'm the executive director of the Kansas Corn Growers Association, Kansas Grain Sorghum Producers Association and also serving in kind of an ad hoc capacity, certainly an unpaid capacity, as chairman of the Triazine Network.

A little bit about the Triazine Network. It was formed back in 1995 as somewhat of a response by growers of over 30 commodities, and certainly, that many states, to provide a vehicle for participation in the US EPA special review of the triazine herbicides. And that

certainly is atrazine, but also simazine.

Our objective is to ensure that EPA has and utilizes the best science. That's why we participate in events such as today. Membership encompasses farm groups from border to border and certainly sea to sea as well.

The executive committee is composed of farm organizations from Kansas, Missouri, Florida, California and Hawaii. We're a very diverse group.

atrazine has been used as the foundation of our weed control programs since the 1950s. It has been around for a long time. It is a very important product. And we know this product well.

We know how to steward it in a way that provides safety for ourselves, or at least we believe we do, and also for the environment that we farm and, probably more importantly, that we live in. We have confidence in the product.

I must say that I will diverge from some of my written comments to respond to a few things that we have seen over the last few days. One of the things that really struck me was at the end of Dr. Hayes' presentation when he placed the slide but he didn't discuss about a corn yield increase of 1.2 percent.

I'm sure in lieu of the discussion that had taken place the

previous four or hours, it was meant to minimize the value of that.

Let me clarify what that means. Number 1, the 1.2 percent we don't agree with. But it is, if you think in terms of percentages, maybe a fairly insignificant number.

But on Kansas Farms, at the farm gate, it is equivalent in corn and sorghum makers to about 120 million dollars a year. That's per year at the farm gate. That's 120 million dollars that's available to support education, medical, ambulances. It is real dollars at the real level. And quite frankly, that is significant where I come from.

I think also it was placed up in the context if you looked at the tail end of the presentation right before then, it was in the context of a lot of different issues that are certainly not any issues to EPA in a special review. Again, we're coming up on the nine year anniversary.

These issues are not new to EPA. Certainly, not even new to the SAP. Previous SAPs have dealt with many of the studies that were laid up as a part of this frog functioning as a canary in the mind type situation.

Many of the studies actually have been addressed and, to some extent, minimized from the original assumptions in the study based on review of previous SAPs.

In fact, the position of EPA today is that atrazine is not likely

to be a carcinogen in humans. I don't think you would have taken that away from the tail end of the previous presentation.

Also, I must comment that I was taken back by the picture of the unhappy frog. I don't know if the frog is happy or not. It was obvious that it was a little bit different type of frog or in a different condition. I wouldn't deny that. I'm not sure what that means. I'm not a frog guy. I don't know these things.

But it just suggested that this was representative of perhaps unhappy frogs in the field, which you would expect after 40 years of usage. And yet, I really didn't hear the case made for that. In fact, I'm not sure I heard a very strong case made for why the very profound laboratory observations and even the specific field observations that were shown and very eloquently presented to the panel, how we could even have frog populations -- the term robust was used earlier, I don't know if I'm qualified to use that term, but how we could have even surviving frog populations if this was, in fact, a legitimate situation in the field.

And that is an issue to growers. All throughout this process, we have seen situations where models have been applied to suggest impacts out in the real world. It's been tough to explain this when I go home and talk to my growers how these situations can exist and yet

they go out behind their house and in the farm ponds that are surrounded by corn and see very, to use the term, robust frog populations and certainly not seeing a lack of frogs or other types wildlife that would be associated with that type of healthy population.

I was also taken back by the link visually between atrazine and DDT. Kind of brings me back to my earlier days in the 60s when everybody was reading Silent Spring. And certainly, I don't mean to minimize the influence of the work of Rachel Carson or certainly the impact of DDT. But I will tell you atrazine is not DDT. I guess I hope I shouldn't need to tell you that. I'm taken back by that kind of sensationalism.

I guess another thing that hits me, here we are, it is another SAP. And guess what, we had another press release come out yesterday on low sperm counts in Missouri folks that had been exposed to atrazine.

Not necessarily new data, but we got a new press release. It has been a while since we have really seen that. I guess we had one at the technical briefing time frame from Dr. Hayes. It was work that had been around since the previous November and presented at SETAC, but all of a sudden it was all over the world news. You could get hits -- a tremendous job of selling the news. Whether it is news worthy or

not, I guess that's for others to decide.

Again, thinking of this new study from Missouri, I haven't had a chance to really look at in detail, but it is interesting to me that the males that were considered viable candidates for the study were partners of pregnant women. That's how they were selected. And yet the study is about low sperm count. I don't know if that makes male men in Boone County, Missouri nervous or not, but I would think it ought to.

To some extent, part of the problem that we see is that the studies conducted by Dr. Hayes in our opinion have led to the SAP. Wouldn't have gotten much more than a snicker if they would have been submitted by a registrant or some other person in the same fashion.

I'm pleased to see that there is a very openness about the data that was suggested today. But I'm also aware of the situation that Mr. Slaughter talked about earlier where when the data was finally released under a Freedom of Information Act request, it was still encrypted.

If you look at the white paper prepared by the agency itself on Page 17, they talk about for these latter studies reviews were less detailed because EPA did not have access through the study office for

the full range of raw data.

You wouldn't assume that based on the presentation this afternoon. In fact, you saw the shelves with reams of data that were available to anyone that really had an interest it.

The last time there was a public meeting that talked about this issue and the agency during the technical briefing April 16th of last year, there was actually a little bit of disagreement whether data was available at that point because it was being referenced in the technical briefing.

In fact, the director of special review and reregistration did clarify to everyone in the audience that they did not have data at that time.

Again, it is good news to hear that it is available now. We expect to be seeing it. But it is also interesting that when the cameras are rolling, the data is readily available, but the historic perspective of this is that the data has not been available. And I think you can attest to it. I don't think you find much of it in your packet.

Dr. Hayes told an audience at Duke University last January in talking about -- I suppose he was looking at the potential for the interaction of different products that he talked extensively about this afternoon.

He used, and I have a quote here, that with other pesticides, the other pesticides "act like frog bullies because they hold them down and let atrazine beat them up."

And I guess my observation is when you are struggling with making your case with good data and good work, at least shared data, you try to give the herbicide in this case an evil personality. And I have a problem with it. I think we have done it a little bit in trying to suggest a link in the same sort of effects between atrazine and DDT.

I would hope that that would be thoroughly discussed by the panel.

Well, in Kansas, it appears that the frog bullies have not been very successful because the frogs seem to be doing very well. I don't mean to be too facetious, but the implications that seem to be negative to a pesticide always have a certain amount of implied truth or believability about them. After all, how could anything that has been used on American farms for 40 years still be good. How could it even be effective.

Well, trust me, there is plenty of competitors in the weed science industry that are looking for alternatives to atrazine. If they were truly out there in all the ways you measure alternatives, they would be used by American farmers.

I guaranty you, one of the companies that would love to have an alternative to atrazine that was really functioning that way would probably be Syngeta. They would be several million dollars ahead. They would be selling a higher priced product at the end of the day. They wouldn't have to deal with the continual types of issues that they have had to.

I'm very happy that they have taken it upon themselves to provide the science with all the deficiencies that have been talked about. They have done more than any other registrant I can envision ever would for any product that is out there.

That's part of my concern as someone representing production agriculture is that if you can't make the case with the kind of science that's been provided on atrazine, what product do you think would ever sustain itself against the types of continuous allegations that are out there?

Now, to be sure if there is a problem, farmers want to know about as much as anyone. They are the ones that apply the product. They are the ones that apply it on where they live by and large. So they have an issue in this, a very serious issue. But they also need to know that sound science carries today, and I think that's what this panel is about.

Most of the times when we testified about EPA positions, we unfortunately have not always been in agreement. Usually, at the end of the day we're getting pretty close. And although it is close to the end of the day now, the issue is not.

I would say that we do think EPA basically got this right. There are confounding conclusions to be drawn. I guess I almost liken it almost a he said she said debate, and it continues even at this hour, where comments are made by one person and then the other person feels like they need to redeem themselves. This could go on forever.

And I think the EPA's position to get established standard protocols is the appropriate way to do thing. Not only is it the legal thing to do if they think there is an issue with effects on amphibians, they don't have a foundation to make regulatory decisions on now, and I know that that's not so much the issue of the panel, but I think you can do a lot to help EPA get this right. Because getting it right is critical. Not just for atrazine, but for all the other products that are out there.

Once atrazine is off the radar screen, other products of course will continue to move forward. And quite frankly, unless we want to see agricultural production move out of this country, we have to come

out with some systems that really can establish the safe use or the unsafe use of those products and move on to others.

Today, I brought three of my compradres up here to help also raise some other issues that they have focused on. With me here today is Stephanie Whalen, president of the Hawaii Research Center, Bill Kubecka, doctor of veterinary medicine from Texas, former president of the National Grain Sorghum Producer Association, current president of the Texas Grain Sorghum Producers.

We originally had a farmer from Boone County, Missouri, that just happened to be from Boone County that was here. He had to leave. I don't know if he has gone back to get his semen checked, but he did have to catch a flight earlier. So we have pitch hitting for him Gary Marshall, my counterpart from the association from Missouri.

Now I ask for Stephanie Whalen to speak first.

DR. ROBERTS: Before we go any further, I think the panel wanted to ask you a question.

Dr. Kelley.

DR. KELLEY: So in the interest of total disclosure, I should tell you I come from an agricultural family myself. My family grows cranberries.

And I know that one of the things that we always think about in

growing crops is we're always looking down the line to the next herbicide or pesticide because regulations change.

Now, in some countries, I understand in Europe, atrazine is not used. I think even in Switzerland where Syngenta is headquartered. I wondered if you are aware of what farmers in Europe used for their broadleaf weeds in place of atrazine and whether we had tried that here in America.

MR. WHITE: I think the European system is just so much different. It is not based -- it is buy and large, if I understand it correctly, which I maybe do or don't, it is based mainly on a level of detection.

There are some situations, for instance, France has proposed a ban of atrazine for corn, but not for grapes. I guess it depends on who has the political power there.

I do understand that there are alternative herbicides that are used that are not, how should I say, they are very similar, in fact, might even be azine but they are not atrazine because of the political climate that has changed that situation in Europe. But it used in some places. It is not a European ban.

But there are some -- some countries like Germany, I believe, initiated more or less a level of detection in groundwater standard.

And because of that, they have moved to other products.

And they also because of those changes are not as competitive in the world market in the production of their commodities. That is a fact.

DR. KELLEY: Thank you.

DR. ROBERTS: Let's move on with Ms. Whalen's presentation. Welcome.

MS. WHALEN: Stephanie Whalen. Today I'm speaking on behalf of our research center, what is formerly known as the White Sugar Planters Association. Just to let you know, I'm representing the sugar industry. We are the research and support organization for that, the Hawaiin industry for the last -- over 100 years.

We have cooperated with the government agencies at the federal and state level in health and environmental studies. And we have a long history of interacting in the regulatory process. In fact, we have been involved in the registration of pesticides even before EPA was formed.

Scientists from our organization were involved with the early work with the triazines. Because the Hawaii soil is different, we did plant and soil metabolism studies for the triazines for our industry.

I just also wanted to indicate, which is not in the handout

received, that we also instituted a voluntary water monitoring program prior to the time that EPA set up the MCL level at 3.

And just to give you a feel for how seriously we take stewardship of the products that we use -- and because we exist, our industry existed on four of the major islands and over 200,000 acres, we felt that it was incumbent upon ourselves to do that work even though it wasn't required at that time.

I won't go over everything I had to say. Some of it is duplicate of what Mr. White has said. Though, I do want to stress the fact again that atrazine is one of the most widely researched compounds, herbicides in history of pesticides.

And like you said, if we can't move forward with atrazine, we don't think the science will ever be there for any compound.

Based on our history of experience with the compound, the sugar industry and the other growers when we formed the Triazine Network, we entered into this process with some level of familiarity of use and time, but also with the open mind that there may be some unknown adverse effects out there and we really need to know about that.

We wanted to be sure that the best science would determine that and that we were committed to accepting whatever the science

reveals.

After all, as Jere pointed out, it we the farmers and our families that are on the frontline of any exposure. And it is our lands that are going to be contaminated first.

So we really are committed to the results of sound science, but based on reasonable demands without political intervention, free of scientific turf battles or special interest agendas.

I think it's of some value for you to understand the process which began, that Jere alluded to, back on November of 1994. And just to give you some kind of idea of the processes we have been following, this panel is a brief but very important part of the continuum from that time.

So far the process has primarily focused on atrazine as was said. But we have been through in this process rumors, information leaks, drafted documents, scientific advisory panels, proposed documents, interim documents, administrative changes, numerous new studies, new laws, the food quality protection acts which came in the middle of this, totally upended the transparent regulatory review process that we started, proposed new cancer guidelines that are seemingly now caught up in bureaucratic quagmire which does affect this process, a lawsuit which allows a single party to dictate the

process, public scares generated by activists and the press, and now scientists' challenges of each other.

We have watched various players, mainly the government, come and go. We've heard about many speculated health effects. More recently, our listening to speculation on ecological effects.

Through all of this, it has been an experience for us, an experience in which we continue to have faith that in the end sound science will prevail through the efforts of impartialed experts such as yourselves and the panel that was heard from yesterday. However, the speculation on human or environmental effects and the timing of the public releases have not ceased to amaze us, and their end does not appear to be on the horizon.

We thought the evaluation and the speculation of the sprague dawley female rat hormone system and its significance for the potential human cancer risk was finally settled after three scientific advisory panels were convened, 1988, 1995, 2000. And now it appears that a fourth will be convened next month on the same issue driven by the NRDC.

We patiently listen and try to fully comprehend the details of the rat endocrine hormone system and its relevance or lack thereof to the human population as we have for the last two days listened

intently to the recent amphibian concerns.

We have asked for assistance from experts. In their rat work, we asked for Canton Health Sciences International to help explain this to us and then to present testimony for us.

In that process, we were upset to find that some of the data from reports that were being used in an earlier document were not available for our experts to review.

It's again troubling that the initiating raw data, this is a subject that has come up already, that has generated many of the studies reported on yesterday and today were generated, the need for this scientific advisory panel, have been difficult or next to impossible to access. And hopefully, as Jere has indicated and Dr. Hayes has indicated that that data will be fully available.

The inability to access raw data is particularly disconcerting to our organization. And the reason that is is that several years ago we were involved in cooperating with the EPA sponsored study contracted to third parties in which we participated through split samples and providing them access to our industry.

The analytical results were not similar. Although, we voluntarily produced our raw data to the agency, they were never able to get the raw data from the party they contracted with, but EPA

published a government report with questionable results anyway, never acknowledging there was industry collaboration and that there was a discrepancy in the split samples.

Our follow-up investigation discovered the laboratory involved had no previous experience in pesticide analysis, and was not required to report raw data in their contract.

I mention this because of the similarity of the current situation and the inherent problems with deciphering and reproducing methods in the open literature. And the fact that EPA has not proposed a validated test system to study is also problematic.

It was not clear yesterday from the various comments that the members of the panel made -- and I'm still not sure that people are clear on the differences on studies conducted under good laboratory practices, because if you have never been engaged in one, you really don't know what it requires, and those appearing in refereed journals.

The purpose of good laboratory practice was to improve the ability to reproduce the study, totally reproduce the study, without having the bodies around to ask at any future date.

So if you have reports that are GLP like, and I don't know exactly what that was supposed to mean, but GLPs are formal regulations that require the submission of all raw data and data audits.

It's like a financial audit, you have auditors of a financial institution, by a nonparticipating party.

I also want to clarify. Scientists don't get certified. I have been involved in GL -- that's what we do a lot of. But we are required to document proof of training. It's the studies that must meet the GLP standards and have quality assurance statements as was indicated before showing any deviation that occurred in how it might affect the results of the study.

I believe -- there is a big difference in reports from the literature, not reports, but studies or papers from the literature which seems to be referred to versus reports that I think we're talking about GLP like reports.

I believe that GLP regulations and complying to that provides a level of confidence way over the peer review system. What bothers me is why the agency does not apply at least a minimum level of these types of requirements to data from reported studies that generate significant concerns such as what we have here today.

I did point out a problem that I saw in the report entitled atrazine Induced Hermaphroditism at 1 ppm in American leopard frogs. I'm not going to go through that. You can read about that. It's really something -- it was very factual data that was recorded

incorrectly and that bothers me in terms of how it influences the rest of the validity of the information, though it might be just being careless.

It bothers me also that this process I described continues to generate endless speculation. First related to the sprague dawley's endocrine peculiarity and now the amphibian endocrine systems.

As scientists, you know a phenomena can be studied for the length of a career with tens of graduate students and many post docs looking at every conceivable hypothesis. Each study leads to more interesting questions to explore. That's great. That's what science is about. And I enjoy that discovery process myself.

However, I think it is important to remember that this is part of a regulatory process, which is expected to be somewhat more pragmatic. There should be a point at which the exploration ceases with a reasonable assurance that a sound decision based on the weight of evidence will be made.

That doesn't preclude that continued discovery and appropriate revisiting of decisions doesn't happen.

It appears from yesterday's discussion, if I understood it correctly, that this process could embark on a whole new area of research, an area in which endpoints, baselines and protocols, and

there seems to be a difference of opinion there, have yet to be defined, because I have heard that some endpoints are very definitely established in the literature, and yet I've also heard that there seems to be some concern. So an area also that seems to be open to many approaches and potentially years of study.

Those of us, and we are the consumers, we are the consumers of this extremely useful product, are looking to you, a panel of prominent scientists, to make decisions based on the existing weight of evidence.

We can all speculate on better ways to redo the less than perfect studies. But how much more data are really warranted? How relevant or significant are the data to the viability of the amphibian populations which have existed in these environments for over four decades and which are now facing reduced exposures through voluntary rate reductions and stewardship programs developed over the last eight years.

We do appreciate the difficulty of your task and we thank you for your willingness to assist the agency in moving forward on this issue. I thank you for this opportunity.

DR. ROBERTS: Before we move to the next presentation, let me ask the panel if they have any questions for you. I see none.

Thank you very much for your comments.

Let's move on to the next individual.

MR. KUBECKA: My name is Bill Kubecka. I am a family farmer from Palacios, Texas. We grow sorghum, rice, cotton and cattle. Again, as Jere previously mentioned, I have served as president of the National Grain Sorghum for two years and currently serve as the president of Texas Grain Sorghums Producers. I am also a veterinarian by education and training.

As a farmer, I value the importance of atrazine. Despite intensive research by weed scientists and makers of competitive products for over 40 years to identify atrazine alternatives, the use of atrazine in herbicide programs continues to provide benefits at a relatively low cost.

But even more important, research shows that without the use of atrazine, yields in our grain crops will drop regardless of the cost. atrazine is the most significant herbicide, especially in conservation tillage programs, and use about 90 percent of those acres.

A point that I would like to bring up and that's grain sorghum is not corn. We face a difficult task in grain sorghum in that we are a smaller crop, much smaller crop, about somewhere less than 10 million acres versus corn of 80 million.

Therefore, there are fewer, substantially fewer weed products available to produce sorghum and certainly fewer alternatives regardless of the cost or the effectiveness.

I think the last time we looked at EPA, we had one product in line to be registered for sorghum. That's all. That's insecticide, herbicide, everything. It's a big issue for us in sorghum even at 10 million acres.

atrazine is the cornerstone of our weed control options. If there are real issues in our health and environmental effects, I think this has been pointed out before, we need to know about them.

I use this product where I live, where my kids, my grandkids live. But I must respond to the reliable information in order to operate my business and be a steward of my land and family.

As a veterinarian, I know that proper care requires a proper diagnosis. And in this case, we don't even know if there is a problem.

It is the position of Triazine Network, and I concur, that this issue should be partitioned away from other issues being dealt with in the completion of reregistration of atrazine.

The agency with the advice and counsel of the SAP should work to approve a protocol and initiate a call-in to help in the further investigation of these alleged issues.

If they believe further investigation is warranted, the EPA should not draw any conclusions at this time despite attempts from activist groups to regulate on the assertion of these studies.

Only one needs to remember previous excursions involving the toxic soup theory I think that was proposed by Tulane, the Cornell monarch butterfly scare and, most recently, the University of California, Berkeley, GMO corn issue in Mexico, to realize that sound science will survive the test of substantial review. But using preliminary science to regulate is not in the best interest of the regulated or the regulator.

DR. ROBERTS: Thank you, Doctor. I don't see any questions. Let's move ahead.

MR. MARSHALL: Good afternoon. My name is Gary Marshall. I am the CEO for the Missouri Corn Growers Association.

Unfortunately, Terry Hilgadick who is a board member of mine was to be here this afternoon and offer this testimony. But his time ran out. He had to catch a flight back to, as Jere said, central Missouri.

I hope that I can adequately postulate some of his remarks as well as add in a few of my own. But I'm going to try to be very, very brief in my remarks.

We have actually three different companies. We have a grower organization, which is kind of a lobbying group, we have a marketing group and we have an environmental group, all nonprofits that work for the Missouri Corn Growers Association. I work as their CEO there.

In addition to that, I have over 30 years of experience in agricultural products and background. I live on a farm. I currently operate a farm. And in a previous life, I applied thousands of pounds of atrazine on thousands of acres of corn in a liquid fertilizer operation that we had for about 15 years.

So I do have some, in fact, probably some extensive background in using the product in central Missouri area.

In the nine years since the special review began, more than 200 studies have been conducted on the safety and benefits of atrazine and have been submitted to the EPA. Question after question about the safety of atrazine to humans and the environment has been answered in a timely fashion by the registrant and growers through the use of sound science.

I would like to add here. I'm really pleased to have the opportunity to participate in this whole process as a grower. It has been nine years now, because I also served, along with Jere and

Stephanie, on the executive committee of the Triazine Network, nine long years that we have been dealing with this issue.

But we're hoping that it finally is beginning to come to some sort of conclusion, we hope.

Over four decades of on-farm use of atrazine has been a very reliable indicator of atrazine in the production of corn, grain sorghum, sugar cane and other crops.

The health and environmental effects of the triazine herbicides have been more carefully studied than any other pesticide group.

Obviously, I'm not a scientist. But I do understand some of the benefits of having atrazine as a tool for crop production on our particular farm where we raise corn, soybeans and we also have cattle.

In Missouri, we have over three million acres of corn. We use atrazine on over 70 percent of that corn, or about 2.1 million acres.

Atrazine allows for good weed control, allows Missouri growers to utilize conservation tillage practices on the overwhelming majority of the corn that we grow today. That has changed a bunch in just the last five to ten years.

But this helps eliminate or even reduce plowing of fields for weed control. Makes cropland then less vulnerable to soil erosion in some cases by as much as 90 percent.

Jere mentioned the cost differential and what it means. In Missouri, we figured that cost differential. To switch to another product, you have two problems. You have a switching cost to go to another product, and there is a significant cost to it. And secondly, you have a yield drop or a yield cost. And we calculate that to be over $20 per acre in Missouri.

If you figure that on our 70 percent of the acres that's treated, that's over 42 million dollars a year. If you want to look nationwide, it is a billion dollar a year difference at a minimum for corn farmers. That's just corn farmers.

It's a significant big product that we use. We use a lot of pounds of it. The loss of this product to some of the smaller grower community would be even as staggering probably as it would be to the corn community.

Missouri farmers are really dedicated to an ongoing proactive approach, to environmental stewardship. And in fact, in Missouri we have what we call the WRASP program. The Watershed Research and Stewardship Program was initiated in 1999.

Since that time we been collecting data on watersheds, and we have best management practices to producers studying the various management practices that we can utilize in a cost-effective manner to

help keep these products on the fields where they belong and not onto water systems.

Since that time the data has been collected in two large watersheds in Missouri encompassing over a million and a half acres of watersheds. And it is really interesting to note as far as the EPA is concerned these two watersheds are now being proposed to be delisted from the 303 impaired water list for the state of Missouri, the 303 D list of impaired water.

So we think that's very significant, and we're hopefully going to be able to take these practices then and move them into other areas of the state and perhaps across the country to help again make our products more effective where they need to be and more cost-effective.

Regarding the frog populations in our area, again, I'm not a scientist, but on my farm we have rivers that run -- one river runs through the farm. Another river runs on the south side of the farm. We have wetlands, one that I have built as a conservation reserve program. We have other wetlands that preexisted there. We have springs, ponds, we have wells.

I can tell you just from my observations the frog and amphibian population appears to be doing very, very well. In Missouri we have

noted no decrease in population that I'm aware of. Nothing has been noted to us by the University of Missouri, the State of Missouri, the Departments of Conservation of Natural Resources.

And, in fact, the Conservation Department is actively pursuing as we have been for a number of years a frog season which in Missouri starts about two weeks from today. So with that, I do hope that your process that you are involved with here does make a difference. Because we are counting on you to work with the EPA registrant and all interested parties to make sure that the information that is presented is of value, it makes sense and we want to move this process forward.

With that, Mr. Chairman, I would thank you for this opportunity.

DR. ROBERTS: Thank you very much for your comments, Mr. White. Is there anything else you or your group would like to say?

MR. WHITE: Thank you to the panel for your time. I know it has been tough. I'm back there where I can get up and move around, but it has to be merciless up here.

Thank you again.

DR. ROBERTS: Thank you, Mr.White. Dr. Kelley would like to follow up on a previous question.

DR. KELLEY: This is a question directed towards Syngenta and also towards Dr. Hayes. I did not see in these Ecorisk Syngenta documents that we were provided as printouts originally the printout of one of the early reports from Dr. Hayes that was supported by Syngenta. And that is on the CD.

I had not read it until this hard copy was just provided to me. But on the CD I could not find the feeding data. If you could provide those to us, one of you, that would be great. Is that possible?

DR. ROBERTS: Dr. Mcfarland, did you want to respond to that?

DR. MCFARLAND: Thank you. I did find in -- for those of you who might want to look for that draft report on the disk that was supplied to the Scientific Advisory Panel, it is in the miscellaneous report section. And that's where that is located.

We did have with us a CD of all the raw data and information that Dr. Hayes provided us on that study that was submitted to EPA previously, and we have it with us here, so we'll print that off and we will be happy to provide it.

When I was scanning through it I wasn't seeing the feeding, though. Anything we have we'll definitely provide. I'm sure Dr. Hayes will be happy to provide that report too.

DR. KELLEY: So the report we have been provided with is a report that was signed off by Dr. Noriega in June of 2000, I think. Is that what it is June of 2000? So that's the one we have been given on our disk. Is that the last one that was received from Dr. Hayes?

DR. MCFARLAND: Yes, that was the last report Syngenta received from Dr. Hayes.

DR. KELLEY: Also accompanying this report are a series of critiques from the statistical consultant, and those are 2002.

DR. MCFARLAND: I believe that was just a summary done in 2002. The statistical report that Dr. Sielkin discussed with Dr. Hayes in his lab were discussed back in 2000. and I think that's the date of the larger report attached in your hard copies there.

DR. KELLEY: Dr. Hayes, in this report you actually looked at sex ratios. In the report you state that there is no effective atrazine on the sex ratios that you observed. And yet in your next study you did get an effect

Could you comment on this discrepancy?

DR. HAYES: Yes, in the report --I'm not sure which report you are talking about.

But in the bigger study that I did, the successful study that I did we reported no effects on sex ratio, however, there were several

animals that were noted in the data that were question marked or that were marked for review.

And these are animals that did turn out and have the same gonadal abnormalities. I have pointed that out to members of the panel. We didn't fully appreciate the extent of the gonadal abnormalities until about November. It would have been after that report was turned in.

DR. KELLEY: In that report you state those were animals that died before stage 66 and they were excluded from the analysis. I mean that's what it says here I just read it.

DR. HAYES: I would have to look at the report and see, but there's -- even on the slide I show, you can see in the sex column from that data set there are animals marked for further review for comment or animals that were question marked.

DR. KELLEY: In this initial study, the mortality appeared to be greater than that which you have in your current study. So you had mortality up to 24 percent.

I think in the -- you had significant mortality associated with ethanol. You did analyze survivorship and there was a mortality in the estradiol treated group of up to 24 percent, but also in the other groups as well.

Do you have a feeling for why your mortality has come down since or what was going on with that initial study?

DR. HAYES: I don't recall that. I have to look at what study you have. The study that I recall where there's high mortality, there was initial study in 1998 where we got high mortality, we terminated the experiment, then we went back and then did the two food studies, which we filed reports on. And then there was a fourth study.

I would have to look at those data to see which study you are looking at.

DR. KELLEY: Okay. Thank you.

DR. ROBERTS: Thank you, and thank you, Dr. Mcfarland and Dr. Hayes.

Dr. Fawcett, I believe is the next speaker.

DR. FAWCETT: I'm Richard Fawcett. I'm here representing the Iowa Corn Growers Association. I appreciate the opportunity to appear before this panel to share a few issues from an agriculture perspective relative to atrazine.

This panel has been charged with the primary task of examining the hypothesis that atrazine could directly affect amphibians. It's also been suggested that atrazine through its known mechanism of action of inhibiting the photosynthesis could cause an

indirect effect on aquatic ecosystems by reducing algae and plant growth. I want to look a little bit at that issue.

If, in fact, atrazine were to have a negative impact on an aquatic ecosystem through reducing photosynthesis of algae and aquatic plants, that means we would have to have currently a deficient level of plant growth.

Quite to the contrary. We're looking at a lot of monitoring data that I will quickly share with you. In the conclusions of EPA's Office of Water, the problem we have with the vast majority of waters in the atrazine use areas, the corn belt primary, our problem is of having much too much aquatic plant growth, not too little.

Looking at the 2000 national 305 B report, excessive nutrients are listed as the most common pollutant affecting lakes, reservoirs, and ponds, accounting for 50 percent of the impaired waters. The other pollutants in order of their occurrence were metals, siltation, total dissolved solids, oxygen depleting substances and last is pesticides.

In the past we haven't had numeric criteria for nutrients to measure impairment of waters. To try to help with that situation, a couple years ago EPA published eco-regional nutrient criteria. We have also had Regional Technical Assistance Groups or RTAGS that

have been looking at the data and developing their own suggestive standards.

The upshot of this is is by the year 2004 states must adopt enforceable standards for total nitrogen, total phosphorus and chlorophyll-a as a measure of plant growth

When you look at those proposed standards and compare it to current monitoring data, which we can see is really throughout the atrazine use areas, the nitrogen, phosphorus, and chlorophyll-a concentrations are routinely two to four times those EPA criteria or the numbers RTAGS have come up with.

For example, if you look at Iowa, we don't have 10,000 lakes in Iowa, but I guess we have about 131, those were monitored intensively throughout a year. All but one of those 131 lakes exceeded the proposed standards for nitrogen and phosphorous, with some being 20 fold above the standards.

Looking at chlorophyll-a, 98 of the 131 lakes exceeded the chlorophyll-a standards.

We have really too much aquatic plant growth in that region, not too little.

Just very quickly, to look -- this is a chlorophyll monitoring data. The standard is that line way down almost at the baseline. You

can see the vast majority of those lakes, the 131 there have far too much algae growth at least as far as the aquatic ecologists are concerned.

Quickly, we can look at the nutrients, the cause of that excessive plant growth and of course the cause -- the detrimental effect that excessive nutrients have is to cause excessive plant growth which then can lead to low oxygen or hypoxia as the plant material degrades or metabolism can reduce oxygen.

These are the nitrogen numbers. You can see that all except one of those lakes far exceeded the nitrogen standards, some with -- tremendous. You see that variability. I think it is one of the possible confounding factors in field studies. I really believe in the field studies with these kind of differences in water quality can certainly have an impact.

That shows the phosphorous concentrations in the lakes. Again, all except one far exceeding the proposed standards.

Excessive nutrients and/or excessive aquatic plant or algae growth are common causes listed as impairments for 303 D lists. For example, in Illinois, 57 percent of impaired waters list excessive nutrients and/or algae as the cause of impairment. In Iowa, 50 percent of the impaired lakes list excessive algae as the cause of

impairment.

Again, we have excessive levels of aquatic plant growth, not too little. Where do all these nutrients come from that cause these impairments?

Part of it in our region where we are we have very fertile soils. I think we may have had higher levels of nutrients in our waters than maybe some of the ecologists believe in the past.

Certainly, agricultural practices can increase the loss of nutrients. The fertilizers we use, the products you put in the land can increase losses of nutrients into those surface waters.

Farmers have been active for years in trying to reduce nutrient losses. Both for economic and environmental reasons. They have adopted a lot of practices to try to reduce nutrients and stop this excessive algae growth. This may be things like conservation tillage that was mentioned earlier.

And of course, by the way, atrazine is really one of the most important tools that let's us use this system. They may be conservation buffers, even putting in wetlands. We heard about that from Gary just a minute ago. Wetlands being designed to try to

denitrifie nitrate.

So farmers understand they have to be as efficient as they can and try to reduce nutrient losses.

But there is a great fear with these standards being set extremely low that farmers may be asked or forced into making even greater reductions that might reduce their bottom line, that might cost more money, reduce yields, and reduce their incomes.

Because with the EPA Office of Water. the message they are getting from that organization is they need to reduce nutrient losses dramatically to reduce the growth of aquatic plants and related problems like hypoxia.

We have almost an opposite message that they may be getting, depending, I guess, how some of this turns out. And if we look at the IRED, there is a low tier risk assessment by EPA that suggests that atrazine in surface water at least could hypothetically reduce harmful or cause harmful reductions in aquatic plant growth, and that we need to stop that.

And that conclusion there is we don't have enough plant growth. We need more.

Well, when a farmer sees those varying messages, they can easily ask the question which one do you want. Which one is true.

They both can't be.

I think EPA will have to be careful to make sure there is a consistent basis and a sound basis for their recommendations.

One other issue to quickly hit on ponds here, farmers have constructed thousands of farm ponds across the country that have really changed the landscape. Most of these ponds have been constructed with federal cost sharing money.

In fact, in Iowa, NRCS says over 80 percent over the years of ponds put in have relied on federal cost sharing money. And while there are very important secondary uses of these ponds, recreational uses, fishing, swimming, that type of thing, in order for that pond to qualify for cost sharing, by law, NRCS must certify that its primary purpose is one of the three things there. Often, it's all three. Grade stabilization for erosion control, flood control or water quality protection of downstream waters.

By design, these farm ponds are constructed at sites that are vulnerable to runoff. Their primary purpose, if you get government cost sharing, and than more than
80 percent of them, the primary process is to trap and process runoff from agricultural land. Runoff that has sediment, nutrients and pesticides.

The presence of a compound like atrazine or a nutrient in a pond rather than being measure of impairment is a measure that is doing what it was designed to do. These were placed there to catch that runoff process and hopefully increase degradation and protect downstream waters.

Farmers are concerned that they have taken land out of production, paid their part for these, they may be penalized in the future regulatory standards are applied to those ponds.

We are almost to the end here. These kinds of structures are very commonly used in watershed protection projects. Here is an example from southern Iowa, Lucas County Lakes Water Quality Protection Project.

The town of Sheriton (ph) uses three reservoirs. As you can see on the top Lake Morris, Lake Ellis and Red haw as their drinking water source. That watershed was evaluated, and NRCS designed a number of structures to protect that water source.

Every one of those little red triangles there is a water and sediment control basin, kind of a miniature little pond. The dotted lines show what we would more maybe traditionally think of as farm ponds.

And if you look carefully, you will see some little blue circles

with a little fountain coming out of the top. Those are wetlands, constructed wetlands at the tops of those reservoirs.

There is more than 50 of those of kind of structures that have been put into that landscape. Again, farmers are concerned, will they benefit in the end or will they be penalized for putting them in.

They do help in protecting that water quality of the reservoirs. But also as a side benefit, they provide aquatic habitat. By the construction of those thousands of farm ponds across the country, we have created aquatic habitat where there wasn't any before, helping to replace some of the wetlands that are lost because of development or agriculture.

And another thing, just from personal experience, we have heard before, but they are full of frogs. I don't know what -- a robust population either. But there is a lot of frogs in those ponds.

From personal experience, I can relate -- on my own home farm in eastern Iowa, we constructed a farm pond almost 20 years ago with government cost sharing money. The thing that surprised us about that pond was the very first year it was full of water. Throughout the whole year we had an unbelievably high population of bull frogs. That pond was surrounded and still is by corn fields treated with atrazine. The frog population remains there.

We have the last overhead. You can applaud for this, conclusions, just in summary, I appreciate being here to talk to you on a busy day. I have tried to talk fast and get through this.

But in conclusion, algae and plant growth in waters in the regions where atrazine is used is excessive. It's not suboptimal. It is very unlikely that atrazine would have a detrimental effect in these areas by reducing aquatic plant growth when we are trying to reduce it already by reducing nutrient losses.

And in fact, atrazine is a critical tool in the systems we use to try to reduce nutrient, sediment and pesticide loss.

Most farm ponds were designed by NRCS to trap and process farm runoff that includes nutrients, sediment, and pesticides. And again, the presence of those compounds in a farm pond shouldn't be a measure of impairment, but a measure of their effectiveness.

And lastly, those farm ponds that we have put on the land have created aquatic habitats where there were none before, and at least anecdotally they have a lot of frogs in them.

Be glad to take any questions if anybody has any.

DR. ROBERTS: Thank you, Dr. Fawcett.

Are there any questions regarding his presentation? I don't see any. Thanks very much for coming and sharing your comments and

views with us.

Well, earlier today, I confidently proclaimed that we would complete the public comments today, but later today I said we wouldn't take any new public comments after 6:30 in view of not getting the panel too rundown.

With that in mind, let's close the session today. There are a few people that are registered to present public comments. I thank them for their patience. And ask them to come tomorrow morning. We will convene at 8:30. We will continue and complete, I say with utter confidence now, the public comments tomorrow morning and then we'll begin our deliberations.

A question in the back?

MR. HEDBERG: I would beg the patience of the panel to take five, about five minutes of their time.

DR. ROBERTS: Are you one of the public commentors that was scheduled?

MR. HEDBERG: Yes.

DR. ROBERTS: If it would be a hardship to present tomorrow and your comments are short, we can take them tonight.

MR. HEDBERG: I would certainly appreciate that.

DR. ROBERTS: I don't think that will be a problem at all. Go

ahead and come forward if you are ready now and introduce yourself to the panel.

MR. HEDBERG: First of all, I thank everybody for your patience. I do have to leave town tomorrow.

My name is Rob Hedberg. I am the director of Science Policy for the Weed Science Society of America.

My comments, the Weed Science Society is pleased to be here regarding this assessment on potential effects of atrazine on amphibians.

WSSA is a nonprofit organization of academic research, extension, government, industry, scientists committed to improving knowledge and management of weeds in agricultural, aquatic, forest, horticulture, range, right of way, natural area environments. Together with our affiliate associations around the country we represent approximately 4,000 scientists.

We're very interested in the special review and re-registration because atrazine as you have heard plays such a major role in weed management throughout much of the nation.

To preface my comments too our impressions that we're going to talk about today are really based on review of the white paper. To the extent that our comments seem critical, it might follow through

with what Ecorisk said, that the white paper was somewhat harsh in its judgement.

However, it's important, I think, at this point to be critical, because the endpoint for the use of this analysis is a regulatory -- it will result in a regulatory decision. So I think it is appropriate to be rather harsh in our critique of the studies.

We would like to make several comments. To provide a new perspective on this discussion, we are basically in substantial agreement with most of the agency's analysis of the studies that were evaluated, but we're not in full agreement with the proposed strategy from our perspective.

I wanted to start with general comments and then respond to some of the specific questions posed to the panel. Foremost, weeds are a very significant agricultural, environmental, and public health problem, and atrazine is a very important herbicide that has been used more than any other compound to control these weed problems.

It has been used on millions of acres every year for more than 40 years.

For most recent years, it has been used on over 60 million acres annually in this country. It has been used in a number of different crops, in different weed management systems, under many

different environmental and climatic conditions.

The sheer magnitude of its use is testimony that this herbicide has provided and continues to provide enormous benefits to many different people.

Because it has been used so widely and for so long, atrazine and its impacts are better characterized, documented and understood than for most other chemicals in the environment. Certainly we know more about atrazine than we do about any of the alternative herbicides that may be available.

Arguably, we know more about atrazine and its impacts than we do about alternative weed management practices whether they be biological, mechanical, cultural type practices.

Based on this extensive history, it is only reasonable and prudent that we should look beyond the laboratory studies to detect, confirm, repudiate, hypothesize adverse effects. Laboratory analysis disengaged from real world validation can give misleading impressions. And this was recently the case when results of preliminary lab studies on monarch butterflies and bt corn pollen were extrapolated beyond their supportable scope.

A great controversy was created, public anxiety was raised unnecessarily and, ultimately, the field studies demonstrated that the

effects found in the laboratory do not correspond to real impacts under real conditions.

We think it is important, this distinction between effects and impacts, because it is the crux of determining ecological relevancy.

In addition to recognizing the distinction between laboratory effects and real world impacts, it is important to keep the purpose of this analysis in mind as we understand it.

This is not a human health issue that we have been convened to look at. We are convened to look at ecological risk.

The human health issue has been addressed by other SAPs and have been, I think, fairly thoroughly considered when the agency issued its Interim RED earlier in January.

These amphibian analyses are being conducted to examine ecological impacts. And as such, they must be to conducted to facilitate the risk benefit comparisons which are required another statue, namely FIFRA.

With this in mind, we feel that any future studies and course of studies as you go forward should be more closely aligned with finding, verifying, quantifying and comparing impacts under real world conditions and with testing hypotheses about laboratory induced effects.

In response then to the specific questions posed to the panel we have the following comments.

Comment on the agency's conclusions about the field experiments inadequacy to ascertain absence of a causal relationship. In our opinion, the field studies did not demonstrate repeatable impacts of significant magnitude to be convincing that there is a real world problem under field conditions. Although this warrants additional examination, and we think we should keep going forward, it appears that field impacts do not even begin to approach the level of concern that would be enough to outweigh the benefits that this herbicide provides.

Especially compelling from our look at the data was the Illinois field study that found only 2.8 percent with a range of 2.3 to 3.6 percent of "intersex" prevalence over three years of sampling in the field. That does not seem like an extraordinary environmental impact.

Question 3A, comment on the agency's conclusions that the laboratory studies provide the basis for a plausible hypothesis about atrazine developmental effects.

Our read of this was that the studies at this point do not offer a good basis for establishing any hypothesis. It appears to our reading that the studies were plagued with multiple deficiencies and

variability.

And our conclusion, much like the agency said, is that we have to some standardized protocols that will yield repeatable results.

One of the things that stuck in one of our reviewers minds was the original Hayes study which triggered this described the containers as being nondescript plastic containers typically used to house laboratory mice.

Well maybe the question had been looked at. But to us, we know there are concerns about estrogenic compounds being released from plastic containers. If you are going to be looking at hormonal impacts, you should definitely qualify what kind of containers are being used and how this was considered in the study.

So I think what we're saying is that these studies are not at a level right now to justify the hypothesis that the agency presented.

Question 3B. We concur with the agency that the variability makes it impossible to ascertain a relationship between atrazine exposure and amphibian developmental effects.

Question 6A. Comment on the agency's determination that there is not sufficient data to reject the hypothesis that atrazine can cause developmental effects in amphibians.

We felt this is a very difficult line of questioning, because it is

more subjective than scientific. Will there ever be enough evidence to prove a negative?

In our opinion the question should be is there enough information to prove that atrazine causes developmental effects in amphibians. And based on the studies which were reviewed, we think that answer is no.

Question 8A. Comment on the proposed sequence of study objectives. We fully support the development and validation of reliable laboratory protocols before any further analysis is pursued. Subsequently, we agree that the original studies indicating possible developmental effects must be independently reproduced before further studies are warranted.

If any developmental effects are found in the laboratory, it would be appropriate at that point to focus further investigation on carefully designed field surveys that can answer whether or not these effects occur in the field environment as well.

Finally, if effects are found in the field, further study should focus on determining whether or not there are significant ecological impacts. Although elucidating the mechanism of any developmental effects is of scientific interest and we would not like to stop that pursuit, we think that impact is what is important for further analysis

in the regulatory scheme.

The final question that we are going to respond to is, comment on the agency's recommendation that X. laevis be used as a primary model.

This is not our area of expertise, but there are some questions which come to mind right away. There are several concerns about the species. The first is its relevance to discerning ecological impacts in North American environments.

Using this species as an indicator of possible impacts in North America would introduce another interspecies variable into an analysis that already seems plagued by unmanageable variation.

Secondly, as documented in the white paper, the species is already known to have a unique hormonal response as to environmental variables which differ from the North American populations.

Finally, as an organization, we're very concerned about invasive species and would not like to see overuse of a species that might conceivably escape into the environment, such that African clawed frogs would become the next northern snake head.

In closing, what we would like to do, the take-home message is that WSSA would like to contrast the absolute certainty we have about

the benefits of atrazine as a weed management tool and to contrast that to the current uncertainty and ambiguity associated with the amphibian risk experiments evaluated by the agency in the white paper.

Although the agency has found the overall weight of evidence so uncertain that does not support any definitive conclusions regarding amphibian developmental impacts, we are absolutely certain that atrazine is a herbicide that provides significant economic and ecological benefits when compared to the available alternatives.

DR. ROBERTS: Thank you, Mr. Hedberg. I think there may be a couple of questions. Dr. Green has one.

DR. GREEN: I just want to make a comment because I would like you to know that we know and are aware of some of the concerns.

I don't want to speak for Dr. Hayes, but concerning the polycarbonate rat cages that you have expressed an interest in here, they actually are designed for use in animal experiments. The kind of plastic that they are does not break down to endocrinological active metabolites.

They were designed for mice because of that exact concern. Over time, though, those will gradually be replaced. In my experience, there are containers that are suitable for housing xenopus

that are designed for use in storing human food. Currently in our facility, that's one of the requirements, that if you are going to house them in something else that is portable, that it is small.

There are sources where you can buy the kind of plastic containers that won't do exactly what you propose they might do.

Your concern about xenopus laevis being an evasive nonnative species is a real one. As you know, that happened in the past, in the early 1980s.

Fortunately, those populations that have escaped and gone to states where they have cold winters and freezing and thawings, as far as I know, those populations have not thrived under those conditions and some have disappeared entirely.

But as a result of that, it takes a state permit in many states now to actually keep xenopus laevis. And I think they will be even more increasingly regulated as we go into it. So those things that you bring up here, those points will be taken into consideration.

DR. HEDBERG: Very good. Thank you.

DR. ROBERTS: Dr. Denver.

DR. DENVER: I just wanted to say that there is some recent evidence that bisphenol-a can actually be released from polycarbonate cages. But nevertheless, that should be constant across treatments.

So one would have to invoke an interactive effect with atrazine. I just wanted to say that.

MR. HEDBERG: This is definitely very far out of our area of expertise, but are there other -- could glass containers be used? I raise this as an outsider questioning the questions that come to my mind. Are there other devices or procedures that could be used that eliminate that question entirely from the analysis?

DR. GREEN: A big concern is the cleanliness and the ability to put things through very large autoclaves and cages washers. And certain kind of plastics don't handle that very well when you do repeated cleanings.

But, yes, there are other types of plastics and other containers that are designed for animal use that we think, although you never know, probably don't release any kind of compounds as they degrade over time that would affect animal experimental results. Glass containers will work as well.

When you have thousands and thousands of animals, though, it becomes very difficult to manage large glass containers, although some people still use them. I think with time, though, things will be replaced to more suitable containers designed specifically for xenopus.

DR. ROBERTS: Dr. Kelley.

DR. KELLEY: Just to respond to the glass. Glass is good, and the frogs like the glass, but steroids stick to glass. So the way you get around it is you coat the glass with something that prevents them from sticking, but then you have some new chemical.

The problems are endless.

DR. ROBERTS: On that note, perhaps we should close the session. I appreciate, Mr. Hedberg, your willingness to come and give public comment this evening.

Dr. Hayes did you have something you wanted to take care of before we closed down?

DR. HAYES: What has been handed to you is not my final report. It doesn't include limb deformities that we reported. It doesn't include snout vent length versus laryngeal regressions that we included. It doesn't include any of the chemical measurements from PTROS or any of the data that were attached to the final report. I will try to track down that.

DR. ROBERTS: If you could try and track down that final report for us, we would be grateful for that. Thanks for clarifying that.

Thanks, Mr. Hedberg, for coming and commenting this

evening.

Thanks to the other public commenters who presented today. It has been a very useful session, I think, for the panel. We look forward to continued comments tomorrow morning and beginning our deliberations. The session is now closed. We will reconvene at 8:30 a.m. tomorrow morning.

(Thereupon, the session was recessed at 6:55 p.m.).

CERTIFICATE OF STENOTYPE REPORTER

I, Frances M. Freeman, Stenotype Reporter, do hereby certify that the foregoing proceedings were reported by me in stenotypy, transcribed under my direction and are a verbatim record of the proceedings had.

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TRIAL DATE:

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