Beyond Estrogens
- Multiple Mechanisms of Endocrine Disruption

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Thank you and good morning. I would first like to thank the Ministry of the Environment for organizing this meeting and Iguchi-sensei for inviting me to speak today.

What I would like to try and do today is to present some new research looking at, if you will, endocrine disruption or looking at wildlife populations and looking at possible effects for the abnormalities that we are seeing that are very different than what I have actually presented in the past. So I am hoping that we can get through this.

As many of you know, we have reported — my laboratory and colleagues — for the last decade that alligator populations and fish populations in contaminated lakes in Florida have a number of reproductive and endocrine abnormalities. These abnormities are especially present in contaminated lakes, which have been contaminated by agricultural activity and high pesticide use.

These abnormities include a variety of effects including changes in hatching rates and mortality of young. In males, we see reduced testosterone, altered estradiol, and reduced phallus size. We also see in females that we have elevated estradiol as a young female, but as these females mature, estradiol is actually depressed and we also have the presence of abnormal follicles in the ovary.

Now, we have studied a number of lakes. Many of you are familiar with the work we have done on Lake Apopka, comparing it to Lake Woodruff. We have also over the last couple of years published a number of studies looking at multiple lakes, and I am going to present some data today on work that we have done over the last four years on Lake Okeechobee and the Everglades in south Florida.

Now lake Apopka has been a lake we have studied because it is intensively contaminated and it had a pesticide spill. But it is important to remind all of you that the effects that we are seeing — depressed testosterone, altered phallus size — are not only seen on Lake Apopka where a pesticide spill took place. We also see it on Lake Griffin and areas of Lake Okeechobee, which just have intense agricultural activity.

First, I would like to actually show you a little bit of data that we have continued to look at these animals but now are trying to get at some of the molecular levels. I would like to thank Prof. Taisen_Iguchi again. We actually have a collaboration going between the National Institute for Basic Biology at Okazaki and the University of Florida. The work that I will show in the next few minutes is actually work by one of my students, Die Bermudez, who has actually worked with Prof. Iguchi over the last couple of months. As you can see, Wani Tai is working here.

One of the things that we have actually been looking at is the expression of SF1, that is steroidogenic factor 1, and aromatase. What we have actually done is collect animals from the wild, these animals that are showing alterations in steroidogenesis. What you see on Lake Woodruff if we compare males and females — and we are looking at relative expression from animals from different lakes — on Lake Woodruff, our reference site, we see females have elevated levels of SF1 compared to males.

Now for those in the audience that work with mammals this is probably a shock, because it is supposed to be the other way around, but in chickens, in birds, and in alligators, this is, in fact, the normal pattern and it, in fact, has been presented previously. Western *et al* actually reported in “Gene” in the year 2000 that this is, in fact, the normal pattern.

What we see on Orange Lake and Apopka, contaminated lakes, is actually no sexual dimorphism, or in fact, maybe even reverse sexual dimorphism, and as many may, in fact, recognize it has been suggested that SF1 may play a role in inducing aromatase gene expression. So aromatase has been cloned out in the alligator. This is the work that Bruce Blumberg and colleagues have performed, and we were able to use their sequence information to make the primers.

What you can see on the reference site where you have normal SF1 appearance activity is dramatic aromatase expression in the female and low levels in the testis in the male. What we find is that there is still some sexual dimorphism that we see on these other lakes, but that sexual dimorphism is suppressed.

To extend this work beyond the lakes in central Florida, we have actually worked in the Everglades in south Florida and we are looking at a number of sites there. We looked at Lake Okeechobee. Lake Okeechobee is the largest lake in Florida. It is, in fact, the water source for all of the Everglades, and much of this water drains down through the grasslands and eventually into Florida Bay.

There are, in fact, a number of interesting sites on Lake Okeechobee. We have a site in the west, which is similar to a reference site: relatively low impact, low nutrients, and low pesticides in the soil. In contrast, Site 2, which is the Kissimmee River drainage, and Site 3, which is Bell Glade, are extensively contaminated from agricultural activity. The Everglades site is actually a restored sugar cane field and has very mixed components of some metals and pesticides, etc.

We have actually looked at these sites and done extensive work both on fish and alligators. I am only going to present the alligator work today.

What I can tell you is that pretty much we are seeing very similar effects to what we see in central Florida. We see depressed testosterone and phallus size in males from the contaminated areas, we see altered hepatic testosterone metabolism, which is not correlated with inductions of EROD, PROD or MROD; in other words, this is not just overall induction of P450, this is very specific alterations in androgen metabolism, and this was, in fact, published just this month in "Environmental Health Perspectives."

We have also seen altered plasma thyroxine levels; we have seen altered estradiol, again low levels in the females; and we see no vitellogenin induction. It goes back to the question which I asked earlier, we believe that because of the large mixture of compounds that these animals are exposed to that vitellogenin is not being presented in these animals because we have not only estrogens, but androgens and possible progestin mimics as well.

What are the causal agents behind these problems? We have spent many years looking at organochlorines, we have looked at heavy metals, but what I would like to do is look at a couple of other components today and show you some very new data on these issues.

The first is an issue, which has actually been in the literature, especially in human health here in Japan, and the United States and elsewhere in Europe for a number of years, and these are the phthalates. However, given all the discussion that has gone on in phthalates, there are, in fact, no data, or very little data, on wildlife exposure.

They are perceived primarily by most people as plasticizers; however, phthalates are also used as chemical stabilizers in pesticide formulations, insecticides, etc. One of the things we do know is there is a very high affinity for many of these phthalates with soil particles. We also know from ecological work done on the chemistry of these compounds in the ecosystem that they degrade very poorly in lake and river ecosystems especially associated with the mud and the muck in these lakes.

We also noted very high exposure can, in fact, produce reproductive effects. What kind of exposure do we have? What we have done is again looked at juvenile alligators.

We went out and caught 50 animals at each site, five different sites. We actually collected urine within five minutes. We are looking at urine because we are looking at the metabolites. This is one way to

get around the contaminant issues associated with plastic syringes, catheters, etc. This is work very similar to that published last year in EHP that comes from John Brock's laboratory at the CDC, and this is work in collaboration with John.

One of the first things we looked at is the mono-ethylhexyl phthalate; it has actually been in the literature. One of the staggering things that we saw initially was that if we looked at the south Florida populations, we found 45 ppm in the urine of these animals. Remember, this is metabolite; this is not, in fact, due to contamination; this is product that went through this animal's liver. We know very little about the metabolism of these compounds in wildlife.

We are finding dramatic elevations in south Florida. In contrast, in central Florida, which includes Lake Apopka, we see relatively low levels, that is, only a few ppm. If you break this down and actually look at the sites in south Florida, we have very large variants. Some animals are exposed to very high concentrations, others low. Some of our animals are dealing with more than 100 ppm mono-ethylhexyl phthalate. Again, it looks like there is very little here, there is relatively little there, in ppm, however.

We have also looked at other metabolites. Again you see that south Florida has higher levels than what we see in central Florida, but note the change in scale for both of these compounds. We are dropping by orders of magnitude. There are a few of these phthalate metabolites that are higher in the central Florida populations than we see in the south.

What is the effect of these compounds? At this point I cannot tell you. But it is surprising, in fact, it was very surprising for us, to see the elevated levels that we saw. This work was with quite reasonable sample sizes of more than 25 animals per site, and all of this has actually been redone and revalidated, and as I mentioned, it has been done at CDC and the chemistry is superb. So where are we with this?

We will continue to try and understand whether there are biological responses in our animals to these phthalates in south Florida. But I also want to talk today about something that is a little bit different, it is something which I do not think has been presented in this forum or any forum talking about endocrine disruption, and that is the possible role of nitrogen pollution in endocrine disruption.

Now nitrogen pollution in the form of fertilizers, fossil fuels, and animals production is, in fact, according to "Science" magazine, just a month ago, the "other global pollutant."

Instead of thinking about this just as a fertilizer that goes in and makes plants grow very well what I would like to suggest is that there may, in fact, be a whole other concern that we have for environmental nitrogen pollution. It ends up that humans are now generating organic nitrogen at about 1.5 times with the natural terrestrial production that occurred throughout history.

A very large percentage of that is based on fertilizers, and much of that generates nitrate and nitrite ion pollution. Most of the aquatic systems that have been examined have been examined in concerns for algal blooms, which is actual primary productivity of the plant mass there. Associated with that we have worried about anoxic, or low oxygen conditions.

In the United States 10 ppm is the allowable drinking water level. There are many agricultural wells that exceed this level and throughout the world there is a growing concern that fresh water systems, well water, and aquifer systems in a lot of developing countries are, in fact, exceeding this level.

Most of the lakes we work on, these pollutants are at part per thousand concentrations — they are highly eutrophic. One of the things we have done is a retrospective study, and I want you all to realize that this is retrospective.

What we were able to do is to go back to our data and look at our plasma testosterone levels in various lakes, that is seven different lakes in Florida, and we were able to compare that — these animals were collected in spring of 1995 — we were able to get nitrogen and nitrate data from the water management district.

If you do a polynomial regression, that is pretty interesting, but it is driven largely by this one dot up here. That one dot happens to be Lake Woodruff, which is, in fact, probably the only non-eutrophic lake that we are studying.

If you do linear regressions, what you find is that there is still a significant relationship between total nitrogen and plasma testosterone in these juvenile animals; it is not very good, but it explains maybe half of the variance. But remember, we have gotten rid of a lot of the variance because we are using both lake means and population means for these two variables.

It is interesting if you do a little playing here. Let us get rid of Lake Woodruff and let us just look at eutrophic lakes by themselves. One of the things you actually find is that there is a dramatic association between plasma testosterone in these animals and total nitrogen on only the eutrophic lakes.

Much more work needs to be done, but we have actually followed this up by doing some experimental studies. One initial study was to look at nitrate. We took juvenile alligator testis, put it in culture, looked at steroidogenesis for 5 hours at 32°C that is our standard *in vitro* culture technique, and we added 10 ppm sodium nitrate. We actually controlled the sodium; that was not a problem. 10 ppm was picked because that is the allowable drinking water standard in the United States.

What you find is that there is a dramatic reduction in testosterone production over a relatively short period of time in the presence of nitrate. This is not unique data. It ends up that a similar report — in fact two reports — have been published in 1999 and 2000 by Panesar, actually showing that in rats the testis exposed *in vitro*, or in fact Leydig cells exposed *in vitro,* or drinking water studies *in vivo,* had the same effect: dramatic decline in testosterone synthesis and circulating levels of testosterone in rodents.

We have also done an *in vivo* nitrate exposure. These were Lake Woodruff yearlings, they are one-year-old animals that were raised from egg, so they have never seen a lake; they were raised in captivity. They were exposed to 10, 100, or 1,000 ppm concentrations. These sound high, but this is actually what we have on some of our lakes. This is a static renewal design daily replacement of water. They were exposed for one month. There are 8 animals per treatment.

We do not have all of the data yet, I just want to show you the molecular part of it. If you look at SF1, what you see is a dose dependent increase in SF1 in the females — that is in the ovary — and that is, in fact, correlated with a dose dependent increase in aromatase expression as well. If you look at the males, sure enough we can get SF1 and aromatase induction by the intermediate dose of 100 ppm.

Now there is a model for this, and I am not going to go in over all of this, but interestingly enough there is a series of papers that have been growing in the literature over the last five years in the medical literature, showing that dietary nitrate and nitrite can, in fact, be converted in the gut and in the blood system via a non-nitric oxide synthase pathway for the generation of nitric oxide.

One of the things that we know is that nitric oxide does, in fact, inhibit steroidogenesis, at least two places that we know to date, which then takes out this whole steroidogenic pathway. Much of this is going on in the mitochondria and the endoplasmic reticulum. We actually can generate nitric oxide, which we know is inhibitory in the testis and the ovary for steroidogenesis, and we can actually do that by using a dietary intake of nitrate.

In conclusion, when we think about endocrine disruption — we all know this — but the product here of the offspring, whether it be at this point or even gene expression in juveniles or adults, is not just the presence of genes, but is the interaction between the environment and the genes, and that is a complex interaction.

To date, we and other colleagues have been able to show that endocrine disruption is not just binding receptors, but that endocrine disruption can work through a number of mechanisms, and those mechanisms and the kinds of products that can induce those alterations are growing. I think we need as a group to continue to focus on multiple mechanisms, as we have actually heard this morning and we have

actually seen yesterday and today, and we have to make sure that we do not just focus on estrogens or on one or two mechanisms. There are multiple approaches.

The work that I presented today is not just mine, of course. I would like to acknowledge Allan Woodward from the State Fish and Game. All of the alligator work is possible because of his collaboration. John Brock, I mentioned, and Prof. Iguchi, and a number of other colleagues were involved in some of the other data. I have had an incredible group of undergraduate students. I mentioned that the molecular data was generated by Die Bermudez. I also have an incredible group of undergrads.

I might mention the beautiful images that you saw are the photographs of two of my very good friends, John Moran and Howard Suzuki. All of it takes money: I would especially like to acknowledge the U.S. Fish and Wildlife Service, W. Alton Jones, and the U.S. EPA for supporting an awful lot of this work.

When we think about endocrine disruption, like this view of the lotus here, this is not the view we usually look at. This is the lotus from the bottom. I think when we think about EDCs, we need to make sure we develop new views and we need to continue to keep our eyes open when we do this work. Thank you very much.

Q&A

Iguchi: Thank you very much.

Daston: George Daston, Procter & Gamble. Lou, I am very interested in the nitrate work. One of the things that we think about with nitrate and nitrite poisoning in mammals is methemoglobinemia. I am wondering whether you have been able to look at that. And not just thought a lot about methemoglobinemia, but also other hemo changes that might explain the tox-sciences paper that you cited, and might be less likely to be as tightly controlled as nitric oxide. I am just wondering whether you have measure methemoglobin in these animals.

Guillette: No, and that is a perfect question. Of course, the reason that the U.S. and other countries regulate nitrate in water is because of methemoglobinemia, and especially in newborns and the use of that water in formula. We have not looked at that in alligators. We are, in fact, trying to look at it.

One of the other things we are doing is also looking at things like metabolic rates, etc., to try and see whether in fact it is not just… we cannot see it clearly, like methemoglobin or hemoglobin changes, we might be able to see it in things like oxygen transport and other aspects of their biology. But that is a very important point.

The other thing I should mention really quickly is that we have actually done one study comparing Apopka animals versus Woodruff animals in their response to nitrate. It does appear to be different. In other words, the Apopka animals seem to be responding in very different ways. So there may be some adaptation, but there also may be some other weird things going on after living your life in high nitrate.

Sekizawa: Sekizawa from the National Institute of Health Sciences. Thank you for a very stimulating lecture today. I am very much interested in your presentation about mono-ethyl phthalate ester detection. I could not recall exactly whether you reported about the level of mono-ethyl phthalate in urine or in the environment of wild organisms, but…

Guillette: It is in the urine.

Sekizawa: In the urine of humans?

Guillette: No, this is in the urine of alligators.

Sekizawa: Oh, OK. I am now pursuing international collaboration to estimate the risks of the ethyl phthalate exposure to humans and also to animals. From what I recall, I also read the paper about LeBlanc *et al* of mono-ethyl steroid esters in USA citizens. From that data I could estimate the regional differences between U.S. citizens and Japanese citizens. It looks like U.S. citizens are exposed to two orders higher compared to Japanese citizens to phthalate esters.

I was wondering what was the contamination source to U.S. citizens. I did not know that phthalate esters are used in pesticides, I thought, especially in the case of di-ethyl phthalate, it is used in medical devices and cosmetics. But it is surprising that your data shows such high urine levels, especially when even the U.S. EPA reported in their ethyl phthalate database that they detected very low levels in the surface water of the ethyl phthalate. So I would like to have your comments on this.

Guillette: Your point is very important. When we did this work, this was done in alligators. We collected urine from alligators. The original data, when it came off the machine, when John Brock and I were communicating, he said, “Oh, there is no phthalates. Do not worry about it. That is one thing you do not have to worry about it in alligators.”

In large part, it was because they were looking at the output in the sense of looking at humans. What we find in humans are relatively low levels. In fact, quite low levels of mono-

ethylhexyl phthalate. There are a few others, especially as you mentioned associated with cosmetics, possibly some other uses that are higher in the U.S. population, as you said, than in Japan.

But wildlife we have not looked at. Most of the ecological data that I know of is actually associated with what is found in the sediments in the lake or water. They are persistent in the sediments. They do get broken down by bacteria, so there is a breakdown, but they are persistent there.

One of the interesting things is that when I started looking into these phthalates in more detail and started looking at ATSDR — basically documents and EPA documents — is that ethylhexyl phthalate and a number of others are, in fact, or at least they have been reported to be, commonly used in pesticide formulations, insecticides, etc. Are they used in herbicides for aquatic use? I have no idea.

That is one of the things we are still trying to find out: what is the source that could give us such high levels in the animals that we are dealing with? They certainly are not getting it from cosmetics, and they are not getting it from plastic devices, at least I do not think they are chewing on baby toys that I know of. So thank you.

Sekizawa: Very interesting, thank you.

Guillette: Thank you.

Iguchi: OK, thank you very much.