Atrazine Produces Hermaphrodites in Frogs: Connecting Laboratory and Field Studies

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Firstly, I would like to thank the organizers for inviting me and also for organizing an excellent meeting. It is certainly an honor for me to be here and share my work with you today.

I am going to talk about some effects of atrazine and show that atrazine produces hermaphrodites in at least one frog species and probably others, and I am going to show a model that tries to connect field and laboratory studies.

I think this model is more important than the effect itself because I think it can be used for a variety of purposes. I will start by talking specifically about the laboratory model, how we used that laboratory model to generate comparative studies, then once we know what to look for in other species, how we do assessments in the field, and then how we use information from the field to generate field simulations in the laboratory, and finally how we use this information to address the impact of field concentrations of compounds in the lab.

The compound I will use as an example is atrazine. This is its structure. It is an herbicide used with monocot crops such as corn, sorghum, and it is also used with stonefruits. It has been used for 40 years. We use in the U.S. 150 million pounds, or 75,000 metric tons annually. It is used in more than 80 countries including Japan.

The model that I will talk about is the African clawed frog, *Xenopus laevis*. Atrazine, as I reported last year, produces intersexes or actually what we are calling hermaphrodites in 20% of exposed animals.

I will show you what the sex looks like. This is a male and a female. This structure is the kidney. The gonad, the testis in the male is short, smooth and unlobed, whereas the ovary in the female is this long structure, it has interspersed black pigment, and is lobed.

In histological cross section, the testes consist of tubules. The ovary has this ring of connective tissue and an ovarian vesicle or hole, in the center. So the sexes are easily distinguished in dissection and histology.

Some of the hermaphrodites, which are produced starting at 0.1 ppb look as follows: here is the kidney, these are two testes, followed by two ovaries, a large testis, and again two ovaries. Histological cross sections along the black lines confirm that these are two testes, here are two ovaries with the ovarian vesicle, a large testis with multiple tubules shown, an ovary, and finally two ovaries again.

We have identified multiple types of gonadal abnormities associated with atrazine starting with 0.1 ppb. These include single-sex polygonadism, or multiple testes. This animal has six testes. These include left-right hermaphrodites with the testes on one side and an ovary on the other; anterior/posterior hermaphrodites, with testes anteriorly, ovaries posteriorly, and the mixed hermaphrodite such as I showed in the beginning. These have all been confirmed by histology and, in fact, up to 20% of the exposed animals starting at 0.1 ppb — probably 40% of the males as I will show these types of abnormalities.

These gonadal abnormalities are also associated with the demasculinization of the larynx or the voice box, which I will not talk about today.

We propose that the mechanism of atrazine action is the following: normally in the testis, testosterone is secreted: it is synthesized and secreted. With atrazine, we believe that aromatase is induced and that estrogen is the result, and the estrogen is secreted from the gonad at the expense of the testosterone, which serves as a substrate for estrogen production.

Again we believe that atrazine produces the effects that we have described by the induction of aromatase, and I will show evidence for that.

Firstly, this is now data from adults that are exposed to 25 ppb atrazine. This shows plasma testosterone from control males from a single study. This shows atrazine treated males. There is a statistically significant reduction in the testosterone level of atrazine exposed males after 30 days and these animals are not different from control females. This is data from one experiment, but we have now replicated three times with four animals each time.

Similarly, we have shown that aromatase is induced. This is the level for control males, which is not different from background, and these are data from atrazine treated males looking at aromatase activity in the *in vitro* gonads.

Those were data that I presented last year, and now this year we have grown some of those animals up to sexual maturity. Now I am going to show you the result of atrazine exposure in larvae if they are allowed to grow for one year.

Normally in a male, this is the testis, this is a default larynx or voice box, focus on this muscle, called the diphteria laryngis, this is the default structure of the forearm, and the default structure of the cloaca. In a normal male, testosterone masculinizes the larynx and the breeding glands of the arm, such that the larynx grows in response to testosterone — there is the larger muscle.

Just to go back: this is the female or default type, this is with exposure to androgen, exogenous or endogenous, you get growth of this muscle and growth of the voice box and the production of these brown breeding glands.

In addition, testosterone is responsible for inducing spermatogenesis. This is a section through the gonad of an 8-month-old control male. This outline shows you a single testicular tubule, so I am following this outline. This outline shows you the normal Sertoli cells with the dark staining nuclei, and this outline shows you the developing spermatogonia. You can see the dark staining sperm heads. That is in a normal male.

With atrazine treatment, again I have shown you earlier that testosterone is depleted within 30 days, and in this case the animals were exposed as larvae; testosterone does not recover. As a result the larynx does not grow. You can see the atrophied structure of the larynx, or atrophied condition as well as the loss of breeding glands or lack of the breeding glands to develop. To show the comparison, a normal male, and then in the absence of testosterone when exposed to atrazine.

In addition, the testis of an atrazine treated male lacks sperm and all cellular structure. You can see a single testicular tubule, but no dark staining sperm heads, there are no nuclei staining in the Sertoli or nerve cells. We have demasculinization of the larynx, the breeding glands, and of the testis associated with the decrease in testosterone. Furthermore, the production of estrogen induces ovarian development and in 20% of the animals, we believe that the estrogen induces a female type cloaca in, again, 20% of the exposed animals.

We have both a combination of demasculinization events with the larynx, the forearm, and the testis, as well as feminization events: the production of ovaries and the subsequent feminization of the cloaca.

The significance of this work — it is a laboratory study and laboratory model — but we have repeated the larval studies five times, the atrazine treatments have been replicated 51 times. This is using multiple sources of animals, and all analyses have been conducted blindly, so everything is color-coded and number-coded, blind to the person administering the treatments and blind to the person conducting the analysis.

We have now taken this laboratory model and asked in comparative studies, do we see these same types of effects in other species, now that we know what to hunt for? We chose a diverse group of species.

This is *Xenopus laevis*, our laboratory model. We also looked at *Rana pipiens*, the leopard frog, and at several species of *Hyla regilla*. We chose them because they represent a broad diversity over amphibians so we can determine if it is truly an effect on frogs or specific to one species.

I am only going to talk about leopard frogs today because of the time.

This shows a normal male and female from the lab. This structure is the testis. This larger structure is the ovary, and the histological cross section — I am going to blow each of these sections up. These show the developing testicular tubules in the male, and in the female you see the ovarian vesicle and the oocytes, or the developing eggs.

Now that we knew what to look for, what is normal in a leopard frog, we decided to do field assessments. This shows a map of the United States, the red areas — this map is courtesy of the United States geological survey — the colored areas show the areas where atrazine is used most. The gray and white areas show areas where there is no atrazine use. This map is based on sales of atrazine.

The overlay shows the range of *Rana pipiens*. They are actually endangered throughout much of this region. The blue dots show areas where we sampled water and areas where we sampled 100 frogs in a road trip where we drove across the United States in July of 2001. I just give you an idea because I do not have time to show you all the sites: this is one of the sites in a non-atrazine use area and this is a site in Nebraska, a heavy atrazine use area — in fact, run-off from a corn field.

These are atrazine levels measured by two independent laboratories, a private and an academic lab. Even levels where atrazine is not used are at 0.1 ppb, a level where we see effects in the laboratory. Levels of runoff from a cornfield can range in the ppm, but in this case on this day when we collected it was 15 ppb.

Again, this is the leopard frog in the field, the animal that we examined. We processed 100 animals from each site — only metamorphoses, not adults. We found at all the sites what appeared to be normal males: so these smooth round structures are testes against the kidney. The ovaries are these long lobed structures, and you can actually see the oocytes, the developing follicles, in the females at metamorphosis.

When we examined histologically what looked like perfectly normal testis, we found the following — this is a magnification, and I will magnify again — these are testicular tubules, but rather than containing sperm, they contained oocytes, as you can see. The question is: is atrazine responsible for this event that we found in the field?

One indication that this is not normal is that animals in the laboratory such as this male do not show this feature. So here is a testicular tubule, these are developing spermatogonia. That is what a normal female would look like developing oocytes. So oocytes should not be in the testicular tubules based on laboratory-reared control animals.

In addition, the red circles show where we find the animals that contain oocytes in the testis. Out of 100 animals, we have never found an animal in this region that has oocytes in the testis whereas at many of these sites 100% of the animals have at least one oocyte in their testis. Having produced an effect in the laboratory that shows interference with sex differentiation and now having shown that the intersex phenomena is not normal and that it, in fact, is only associated with atrazine use, we believe that atrazine may be at least partially responsible.

The next step is that we are now conducting field simulations. At these sites, as well as these sites, we have had chemical analysis done of all the suspected pesticides used on the fields. This shows one such example from a cornfield. This shows our threshold level for sex differentiation problems. Atrazine and metolochlor are used in combination and are persistent at least in this one site that we have examined, but all of the other suspected chemicals in that pond are undetectable in the water.

At this point, we are doing laboratory simulations that examine these levels in the laboratory. Again we have taken this laboratory model, we have conducted comparative studies to help us determine

what is normal in these other species as well as treatment studies in the laboratory for these species. Then, we have conducted field studies to determine if the effects observed in the lab occur in the field. Then, we have conducted field simulations, which are ongoing, to determine if the levels determined in the field at sites where we see abnormalities will produce abnormalities in the laboratory.

In our final experiment, where we literally bring the field back to the laboratory, we are conducting the following: at multiple sites, such as this cornfield in Nebraska and this control site in Wyoming, we have collected more than 200 5-gallon buckets of water from each site. That water was then shipped back in a transport vehicle that held the water in the buckets at -20 degrees. We are now conducting studies of this water in the laboratory to determine if it will produce effects such as these already identified in our laboratory model.

I think what is important is that we have identified a compound that may have detrimental effects on amphibians, but also a compound whose mechanisms are common across vertebrates and may be indicative of negative effects on other vertebrate groups as well. Also, I think we have generated a model where we can truly assess the effects of individual compounds or mixtures of compounds in the laboratory. Alternatively we can even start here to allow us to predict ecologically relevant mixtures and test those in the laboratory.

Finally, before I complete, I would like to acknowledge everyone in my laboratory who has participated in these studies. The people in blue are all bachelors, are undergraduate students. So we have had involvement with students at a very young age. I would also like to thank all the groups that have funded the work: National Science Foundation, Howard Hughes Program, Alton Jones Foundation, World Wildlife Fund, and the Rose Foundation. I would also like to thank all of the Fish and Game, Wildlife and Natural Resources Departments that have helped us with collections. I thank my family, and also you for your attention and your time. I will address any questions now. Thank you.

Q&A

Iguchi: We heard textbook talk about verification while going back and forth to the field and laboratory. Does anybody have a question?

Kawasaki: My name is Kawasaki, and I am with the Environmental Monitoring Laboratry in Osaka. Thank you for a very valuable presentation. We are analyzing atrazine at our laboratory. We are particularly involved in analyzing river water and tap water. We have detected the 0.1 ppb concentration you mentioned in tap water in Japan. You said in your presentation that abnormalities have been observed in frogs at a concentration of 0.1 ppb. I would like to know what the period of exposure was when these abnormalities were observed. I would also like to know if in addition to atrazine, you are studying other triazine pesticides such as simazine.

Hayes: The question is regarding atrazine at 0.1 ppb. In the studies where we see the hermaphrodites, those animals are exposed throughout their larval period. They are exposed from hatching until metamorphosis, which in *Xenopus laevis* ranges from 6-8 weeks.

You are correct that in tap water and in ground water, levels can exceed 0.1 ppb, and even in rain water levels can be 1 ppb. There are some studies in Iowa showing levels of 40 ppb, and the tap water in Nebraska certainly exceeds 0.1 ppb. So we do think that it is a real risk. Does that answer you entire question?

Kawasaki: Are you studying other triazine pesticides such as simazine? or not yet?

Hayes: Right now, we have done some studies with simazine, which is used in California. We have completed those dosing but have not yet analyzed the results. We are also studying at least three of the atrazine metabolites that are very common. It is also not known if the metabolites themselves have activity.

Kawasaki: Thank you very much.

Pickford: Dan Pickford, AstraZeneca. Thanks for a very interesting talk Tyrone, One question I have is, in the pictures you showed of the atrazine exposed *Xenopus* metamorphosis, where you have the various kinds of intersex or hermaphrodite, you are describing them as multiple testes.

I am interested in why you call them multiple testes rather than a fragmented testis. And have you looked at — and I know it is easy to come up with other ideas — but have you looked at differentiation of the gonad earlier in development before the metamorphic stage which you are looking at. Have you tracked it back to see whether you have actually got individual testes developing, or whether it is actually starting normally and then it is fragmenting? I am interested in why you are calling them separate testes, not just a single testis which is abnormal over the length of it.

Hayes: So the question is why we are calling them abnormal testes…

Pickford: One multiple testis. Are they different testes or is it just one testis that has sort of fragmented during its development and you have got areas as well as just not development of the tissue.

Hayes: The answer to the first part is that we have not looked earlier. All of the animals that we have examined were at stage 66 or at the final stage of development.

The answer to the second question is that we have conducted the histology of the animals, so if you look at a sagittal section through here, and then if you look at a sagittal section through this group, the testis that we are calling multiple testes are not, in fact, connected. For example, if you look at this structure, there is no connection between those two testes. If you look at, between here and there, there is no connection, there is no

duct between those gonads, and that is why we refer to them as multiple gonads.

I do not know the mechanism of whether or not sections have been deleted or sections have simply never developed. We have not looked that far yet. I do know that this is also induced by estrogen during very early stages of development from some work I did about five years ago, which is what led us to want to look at, in fact, the aromatase induction to begin with.

Pickford: If I can ask another quick question: with the *Rana temporaria* in Europe and the U.K., myself and Prof. Cruse find that if you are looking at — there is not really an equivalent to stage 66 — but in metamorphosis the degree of gonadal differentiation does not seem to be as high. There seems to be a much higher incidence of ambiguous gonads or intersex when you are looking at larvae that just finished metamorphosis. In your lab assays, have you found that in your controls, and does that have any influence on what you are able to detect with atrazine in *Rana*?

Hayes: What we did, the way that we identified these things as abnormalities is we went blindly through over 1,000 animals. I, then, wrote down everything that I saw. So for example, long unpigmented testes like these — I will not bother to show you — ovaries that lacked pigment…

Then we decoded everything. Anything that we saw of equal prevalence in controls and in atrazine we did not call an abnormality. So all of the things that I am listing as abnormities are things that never show up in control animals, the multiple gonad types, etc.

In the case of the *Rana*, there is definitely much less variation right at metamorphosis, and we are now extending those studies to raise the animals for one month. The only thing I can tell you at this point is that we do not see the oocytes in the testicular tubules in lab-bred animals, and we do not see the oocytes west of Nebraska.

Pickford: Thank you.

Iguchi: Any other questions? Two of you. Okay, go ahead.

Tamura: My name is Hiroto Tamura from Meiji University. In the first phase of the detoxification process, P450 plays an important role. My question is whether this induction of aromatase is specifically induced by atrazine or not.

Hayes: Is it specifically induced by atrazine…?

Tamura: Yes. The P450 is usually induced by all foreign chemicals in our body to detoxify compounds. So you find atrazine induced the aromatase. Aromatase is also P450.

Hayes: Yes.

Tamura: So then, is this induction specifically induced by atrazine or not?

Hayes: I am not sure if I am understanding the question, but in atrazine exposed animals, the biochemical assay we use, the 1- β atrazine tritiated water assay shows that the atrazine does, in fact, induce this activity which we never see in controls. We are working now with someone to look at the mRNA for the aromatase screen, but right now, I do not have those molecular data.

All I can tell you is that the biochemical activity is induced by atrazine and never seen in the gonads of control animals — at least in our laboratory. Does that answer you question?

Tamura: …maybe at floor. Thank you.

Q: May I ask a question in Japanese? In the case of outdoor exposure, it is thought that sex reversal is more likely to occur than intersex. Did sex reversal occur in your experiments? I assume you studied about 100 individuals. Could you tell me if you checked if the sex ration changed?

Hayes: The question is I believe, are there changes in the sex ratio? No.

We see males or females or these hermaphrodites. However, if there were animals that completely transferred, some small percent, there is no way that we have right now to determine that there are, for example, sex-reversed females, or males that have been converted to females. We either see males, females or hermaphrodites. Some of the females may be genetically males. There is no way that we know right now to determine if that is the case. We do not see statistically significant shifts in the sex ratio other than the production of hermaphrodites.

Q: Thank you very much.

Q: My name is Kadokami from Kitakyushu. I have a question in Japanese.

We are also studying such amphibians in Japan. Just as Prof. Uchiyama stated in his presentation yesterday, we have found some testicular oocytes. We also believe this may be due to the influence of chemical substances. I would like to know if you could tell us, if you know, what the rate of occurrence of testicular oocytes is in amphibians from the so-called natural environment where there is no influence of chemical substances.

And if so, what possible causes are there in the natural environment, other than chemicals?

Hayes: In the case of *Xenopus laevis*, I have no data on the incidence of hermaphrodites or intersex in its natural environment. I know that in over 10,000 observations per year, we never see these gonadal abnormalities in control. In the case of *Rana pipiens* in the single study that we have done, looking at 100 animals, 1,000 animals across the U.S., I know that the testicular oocyte does not occur west of Nebraska, west of where they use atrazine.

I know that it occurs in Iowa in 100% of the males that we collected. I know that it occurs at least one site in Nebraska at 100% of the animals where we collect it and at smaller percentages in the other areas, but not west of atrazine use, not west of Nebraska. I do not know what else might cause it — other estrogenic compounds. These other abnormalities are all inducible with low dose estrogen which we have shown some time ago. Thank you.

Kadokami: Thank you.

Iguchi: You have a question? We have time for one more.

Q: If you have any data on how reproduction is affected in individuals in which intersex or testicular oocytes occur, could you please share it with us?

Another thing I'd like to know is individuals exposed in the field, other than abnormal sex differentiation in the testicles —I don't think it would be the estrogenic effect of estrogen or atrazine — could you tell us if there were other effects or abnormalities?

Hayes: Part of the question is how does it affect reproduction. And part of that answer is these one-year-old animals, one-year-old *Xenopus laevis* in the lab, we are actually now pairing the males with females and looking at fertility rate, and we do have some data, but because we have only done it once with five animals per treatment, I am unprepared to discuss those data right now. We do have some indication of an impact on fertility. We are also studying the vocalization as well.

In the case of the animals in the wild, I, of course, have no data. But in the transect that we made, there are other abnormalities identified in some populations: so the incidence of parasite exposure and the incidence of limb deformities is very high west of Nebraska: just the opposite of the effects on intersex. There is a much higher incidence of limb deformities and of parasite exposure in the kidneys etc., for example, from Utah and Wyoming where there is no atrazine. But those are the only data I have at this point.

Q: IGB Germany. Just a short question: I am wondering if you are also using *Xenopus* as a lab model? And your hypothesis is that aromatic

atrazine is inducing an increased level of estrogens, but I wonder whether this is true because it is well known in *Xenopus* that you will induce feminization and shift of the sex ratio towards feminization. So if your hypothesis would be right, you should also get an increased number of female phenotypes at least. So I am a little bit... maybe the hypothesis should be extended.

Hayes: Yes. My proposal is that the estrogen that is produced by the induction of aromatase probably at stage 52-54 is at a stage where it is not effective at producing 100% complete conversion, and those data are based on the fact that if we expose animals to low doses of estrogen along the same stages when the gonads differentiate — stages 52-54 — we see these exact same types of abnormalities. So if we give animals below 1 ng/ml estradiol, only between stages 52-54 we see the polygonadism and the hermaphrodites, but not complete sex conversion.

Iguchi: OK, thank you very much.