Protecting Life
From Research to Regulation

Disappearance of frogs, human health effects linked to pesticide use

The African clawed frog (Xenopus laevis). Photo by Brian Gratwicke, December 30, 2012.

Editors Note: There are few scientific research projects more important to protecting life and preventing its long-term demise than those conducted by Tyrone Hayes, Ph.D. And now this work is under threat. Dr. Hayes, a Harvard educated biologist and professor of Integrative Biology at the University of California, Berkeley, whose research finds that the herbicide atrazine feminizes male frogs, is one of the leading scientists critical of the pesticide industry and regulatory process. This critical research is threatened while, as Dr. Hayes' points out, amphibian species are in decline and they are disappearing. Dr. Hayes' work has shown that current regulatory reviews allow widespread use of pesticides that cause serious adverse effects well below allowable legal standards and when in mixtures not studied. He initially began his research with a study funded by Novartis Agribusiness, one of two corporations that would later form Syngenta, the maker of atrazine. When his results contradicted Novartis' expected or desired outcome, he was criticized by the company, which withdrew its funding. Dr. Hayes continued the research with independent funding and found more of the same results: exposure to doses of atrazine as small as 0.1 parts per billion (below allowed regulatory limits) turns tadpoles into hermaphrodites – creatures with both male and female sexual characteristics. When his work appeared in the prestigious Proceedings of the National Academy of Sciences, Sygenta attacked the study, starting an epic feud between the scientist and the corporation. In fact, a June 2013 investigative report by 100Reporters and Environmental Health News exposed the chemical giant's multi-million dollar campaign to discredit atrazine critics.

Dr. Hayes has since published more than 40 papers, over 150 abstracts and has given more than 300 talks on the role of environmental factors on growth and development in amphibians. With the cutbacks in government funds and the relentless industry attacks, Dr. Hayes has recently run into financial woes, including exceedingly high fees from the University's Office of Laboratory Animal Care. We hope that his important work will continue, however without funding there is no way for him to do research. The following are edited excerpts from Dr. Hayes' talk at the 31st National Pesticide Forum held in Albuquerque, NM. You can watch his full presentation online on Beyond Pesticides' YouTube Channel: www.youtube.com/bpncamp.

We have established The Fund for Independent Science to support Dr. Hayes' work, to protect life from harmful chemicals. Dr. Hayes' lab operates on a budget of $150,000. Funds raised will keep this critical research going forward. Contact Beyond Pesticides to donate, or go to www.beyondpesticides.org/fundscience. —Jay Feldman

By Tyrone Hayes, Ph.D.

Tyrone Hayes, PhD at the 31st National Pesticide Forum in Albuquerque, NM.

I study frogs. My work focuses on the role of hormones in frogs. I got thrown into this pesticide thing initially because I got asked by Novartis (merged with Zeneca Agrochemicals to create Syngenta AG) to study its herbicide atrazine, a weed killer that is mostly used on corn in the U.S. It's been used since 1958. We use 80 million pounds a year. It's used in more than 80 countries and is outlawed in all of Europe. The company asked me whether or not atrazine interferes with frog hormones, because I have extensively studied frog hormones. I was specifically asked to use the African clawed frog because it is the frog that everybody uses in the laboratory to study development. In 1920, a researcher discovered that the human pregnancy hormone, human chorionic gonadotropin hCG) would make this frog lay eggs. By 1940, the pregnancy test identified this hormone. I tell this story for a couple of reasons. First, it shows the value of basic research. Second, it shows you how similar our hormones are to frog hormones. Chemical exposures that affect frogs are very likely to affect us—albeit at different doses, and maybe through different routes of exposure.

Laboratory Research

While working for Novartis, we discovered that atrazine decreased, or inhibited, the growth of the voice box, or larynx, in male frogs. This is bad news, since males have deeper voices because of testosterone. For this same reason, male frogs sing while females don't. Data suggested that atrazine is somehow decreasing testosterone and demasculinizing —or, I like to use the term "chemically castrating"— these frogs. We did some very early studies where we looked at the gonads and asked what might be the cause of this decrease in testosterone. We found that some of these individuals, when exposed as tadpoles, had ovaries, then it has another testis, and then it has more ovaries. No frogs are naturally hermaphroditic. So we proposed that atrazine turned on aromatase, which is the enzyme that converts testosterone into estrogen. The idea is that, when males are exposed developmentally, their testosterone is being used up. This demasculinizes them and their larynx doesn't grow. They are now feminized, because they are making the female hormone and will grow ovaries.

In that first early paper, we were able to show that if you expose frogs to atrazine, their testosterone levels drop to those of a female. The paper, *Hermaphroditic Demasculinized Frogs after Exposure to the Herbicide Atrazine at Low Ecologically Relevant Doses*, was published in the *Proceedings of the National Academy of Science (PNAS)* in 2002.

As important as it was for my career and for figuring out what atrazine was capable of, I still had a few questions that were left unanswered. For example, we didn't know if hermaphrodites were males with ovaries or females with testes. And, we didn't know what happened when these animals grew up. The problem is that frogs don't have sex chromosomes, so it's difficult to know who is who, and it takes these animals about four to five years to grow up. So, you have to convince a first year undergraduate and say, "You know, maybe by the time you graduate we might have something for you to publish." We know the answer now. In fact it took us eight years to figure it out.

Sex Change and Behavioral Changes

We discovered that when some of these hermaphrodites grow up they actually completely convert to females. After eight years and the publication of our work, we had identified a gene that is expressed in females that does not exist in males and we figured out that about 10% of the males that are exposed to atrazine completely turn into females. But I also wanted to know what happened to the other 90% of the exposed males. So we did these real simple experiments that I call the "pool party experiment." These are experiments where we put together females with four unexposed males and four atrazine-treated males. The idea was to see if these guys could compete. We put them in the pool at 7:00 p.m., the lights go out, and then the next morning we just look at who got lucky and who didn't.

It turns out, when you do these trials over, and over again that the atrazine-treated males almost never win. Even though they didn't turn into females, they're not competitive. I'm an endocrinologist;

I study hormones. So, I have to do more than observe their behavior. We measured their testosterone levels and, as you might guess, the controls have a lot more testosterone on average than the atrazine-treated males. What's more important, if you look at who made the love connection in these trials, it turns out there is a kind of threshold and most of these atrazine-treated males just don't have the testosterone. We didn't know if that means the females don't like them or the other guys just beat them up. All we know is that by the time the morning comes these guys with the low testosterone lose.

Then we did another series of experiments that I often call the "Motel 6 experiments." In this case, we just put animals alone with females and ask, can you perform in the absence of competition. We know you're not competitive, but are you competent? The way we measured competency is by leaving them alone in the motel room overnight and then we collect the eggs and just look at how many eggs they fertilize. When you do that, control males fertilize about 85% of the eggs, while atrazine-treated males fertilize only about 15%. There is a clear difference in their fertility. They not only are not competitive, they're not competent.

It turns out they're not competent for two reasons. One is that they don't even try. If you observe them, they sit there and watch the female lay eggs. In addition, if you look at their testes under a microscope, you find that the control males are full of sperm, whereas the atrazine-treated males have testicular tubules that are basically empty with a little bit of cellular debris. They don't have enough testosterone to show behavior and, even when they do, they don't have enough testosterone to maintain their sperm.

EPA Ignores Original Research

Then we asked some other questions. "Is this just specific to African clawed frogs or might all frogs be susceptible to atrazine?" We looked at North American leopard frogs and we actually found eggs bursting to the surface of the males' testes. Now at this point I started interacting with the Environmental Protection Agency (EPA). I sent this [research] to the EPA, and they wrote back, "Well, this is an interesting finding Dr. Hayes, however we do not think it is an adverse effect that would trigger review and regulation of the chemical."

Field Research and Multiple Factors

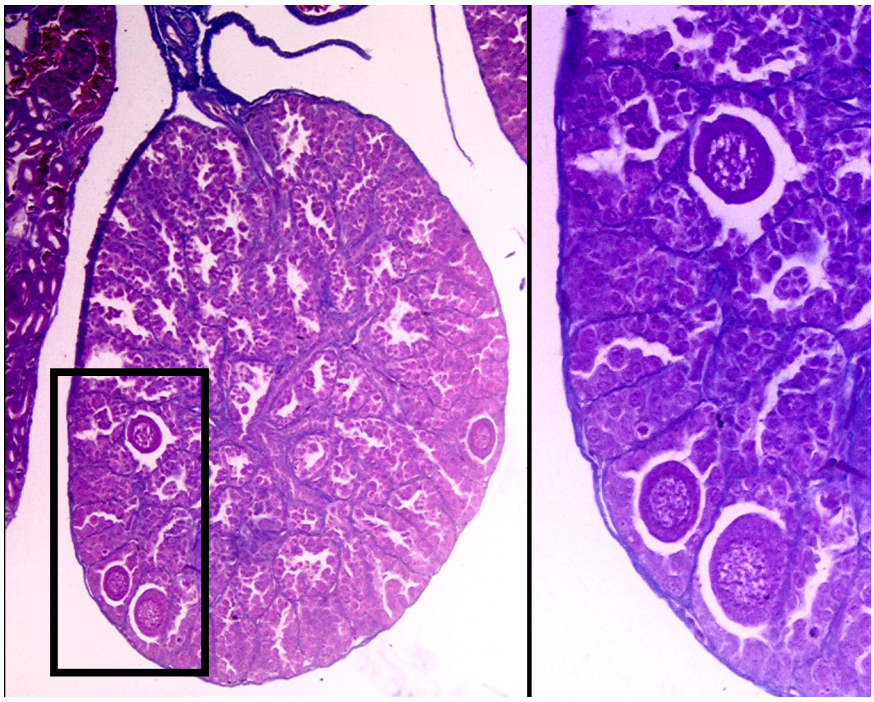
The next thing we wanted to know was whether these effects occur in the wild. Figure 1 shows a testis of an animal collected from the wild, including the testicular tubules with eggs instead of sperm. In the real world, these animals are growing eggs in their testes instead of sperm, just like we see in the laboratory.

It turns out that every place we find hermaphrodites we find atrazine, and vice versa. The reason this study got published in *Nature* is we had the lab data to back it up. We knew we could take frogs from nature and raise them in the lab in clear water and they wouldn't become hermaphrodites. And, we could take frogs from nature and put them in atrazine and know that they would become hermaphrodites. So we knew it wasn't a natural variation, and we knew it was more than a correlation.

We want to ask, how important is atrazine? That is what we are focusing on, but how important is it really? I was concerned because they're not just using atrazine in typical field conditions, they're using all these herbicides, fungicides and insecticides.

In the study, we found that there are multiple factors that can affect development. If the temperature goes up, that's a stressor. If a pond dries up, that's a stressor. If the tadpoles become crowded, that's a stressor. Agriculture contributes to climate changes, contributes to desiccation, and loss of surface water, which leaves the animals crowded. That crowding causes an increase in stress hormones. We also show that mixtures of pesticides, which concentrate as the pond dries up, contribute to an increase in stress hormones and that causes a release of pesticides stored in fat, which effectively increases stress hormones even more.

Figure 1



Transverse serial cross section of leopard frog's testes. Slide image courtesy Tyrone Hayes, Ph.D.

There is this incredible nasty interaction of effects that results in damage to the thymus, or to the immune system, which causes animals in the lab to develop meningitis. The response to a flagella bacterium causes high parasite loads in the kidney and liver in the field. If I didn't have the lab data and the field data, you would never guess why these frog populations were disappearing. You would think it was disease. But in fact, pesticides and other stressors are playing an incredible role in terms of determining how susceptible the animals are to disease. By damaging the kidney and the liver, you're effectively increasing the pesticide load because now you've damaged the organs that are supposed to metabolize and get rid of the pesticide.

Figure 2

Tadpoles from different sections of the Salinas River. Slide image courtesy Tyrone Hayes, Ph.D.

We wanted to test this experimental paradigm more in the field. We were able to do so in the Salinas River. The river flows south to north with most of the agriculture up in the north, creating an incredible experimental regime. We could go to the Santa Margarita where the water is all nice and pristine, a foot and a half deep and 20 degrees Celsius. We can go down river where there is no water because it's all being drained off for agriculture use downstream. Here the tadpoles don't have pesticides, but they're crowded, they're hot, and they're stressed. Further downstream, the water is back at a foot and a half deep, 20 degrees Celsius, just like before, except that 100% of that water is agricultural runoff.

We did a really neat experiment where we started upstream and collected tadpoles at those three sites. Figure 2 shows three tadpoles at the same developmental stage, same age, same species, same river, collected on the same day, about two hours apart. The only difference between the two smaller tadpoles is that the first smaller tadpole is from one of those little crowded hot pools. The only difference between the second smaller tadpole and the larger tadpole is that the smaller tadpole is living downstream of water that is running off the food that we're eating.

Amphibians in Dramatic Decline

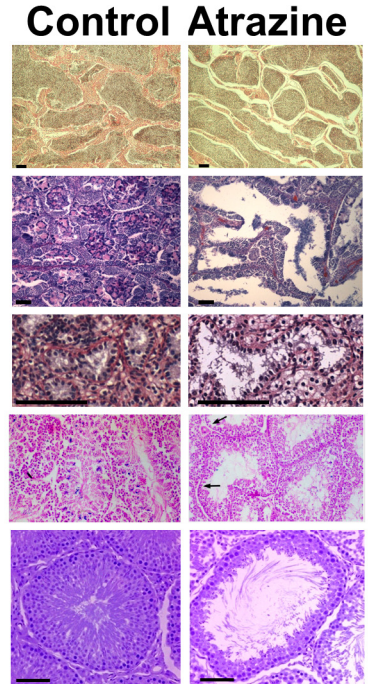
Over 70% of all amphibian species are in decline. This is a group of animals that have been around since the days of the dinosaurs and we are losing species now faster than the dinosaurs disappeared from earth. This sixth mass extinction will be the first time that a mass extinction on earth will be caused by a single species.

Now, what I have told you about is more than one species, or more than one population, that multiple species generate families of frogs. And, I have told you about more than just correlation. I've told you about experimental evidence supporting the impacts of atrazine and pesticides on amphibians and their declines. What's more, though, it's not just frogs. I'm going to show you that there is data in fish, birds, reptiles, and mammals, including humans, that show very similar things happening. The data that I'm going to show you comes from a group of scientists with whom I've worked. I emailed everybody in the world who has worked on atrazine and we've written a couple of papers.

Reproductive Failures

We published with 22 authors from 12 different countries. My frog has sperm in the testes. Give them atrazine, no sperm. A scientist from Belgium with fish sperm in the testes; give them atrazine, no sperm. With reptiles, sperm in the testes; give them atrazine and they look just like my frog. Rat studies done in Croatia and

Figure 3

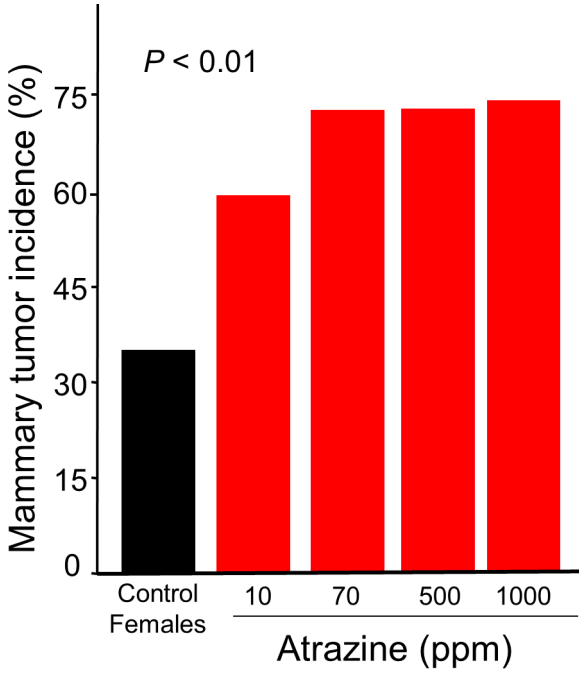


Testes of multiple species exhibited egg production after atrazine exposure. Slide image courtesy Tyrone Hayes, Ph.D.

Nigeria show the same. This is all peer reviewed published data. Testicular tubules with sperm, give them atrazine, no sperm. A new colleague from Pakistan has shown that you take quail, with sperm in their testes, give them atrazine, no sperm. It doesn't matter what animal you're looking at, the same thing is happening in the tests. The route of exposure might be different, and important concentrations might be different, but the same effect occurs. It doesn't matter if you're looking at fish, amphibians, reptiles, birds, or mammals. (See Figure 3.)

Of critical importance is that my colleague Shanna Swan, Ph.D. at the Icahn School of Medicine at Mount Sinai found what she calls sub-fertile men in Columbia, Missouri. These men have about .1 parts per billion atrazine in their urine. These men, who have a low sperm count, have about as much atrazine in their urine as it takes us to chemically castrate a frog. Now that's just correlation. But, remember that atrazine knocks out testosterone in sperm in fish, amphibians, reptiles, birds, and rats, which are mammals like us. What's more is men who apply atrazine have 2,400 parts per billion of atrazine in their urine. That's 24,000 times higher than we know is associated with low fertility with men in Columbia, Missouri. Men who apply atrazine have 24,000 times the atrazine in their urine that we use in the laboratory to chemically castrate frogs and fish. If one of these guys were to pee in a bucket, I could dilute it 24,000 times and use the atrazine in their urine to chemically castrate and feminize 24,000 buckets of 30 tadpoles each.

Figure 4



Syngenta study found cancer in female rats increased after exposure to atrazine. (Stevens et al. 1994) Slide image courtesy Tyrone Hayes, Ph.D.

Disproportionate Effects

Applicators are often part of a segment of our population for whom we know there is a health disparity. Black and Hispanics are at greater risk and usually have poorer outcomes and are more likely to live and work in areas where they are more likely to be exposed to chemicals that we know are associated with these same illnesses.

Atrazine and Cancer

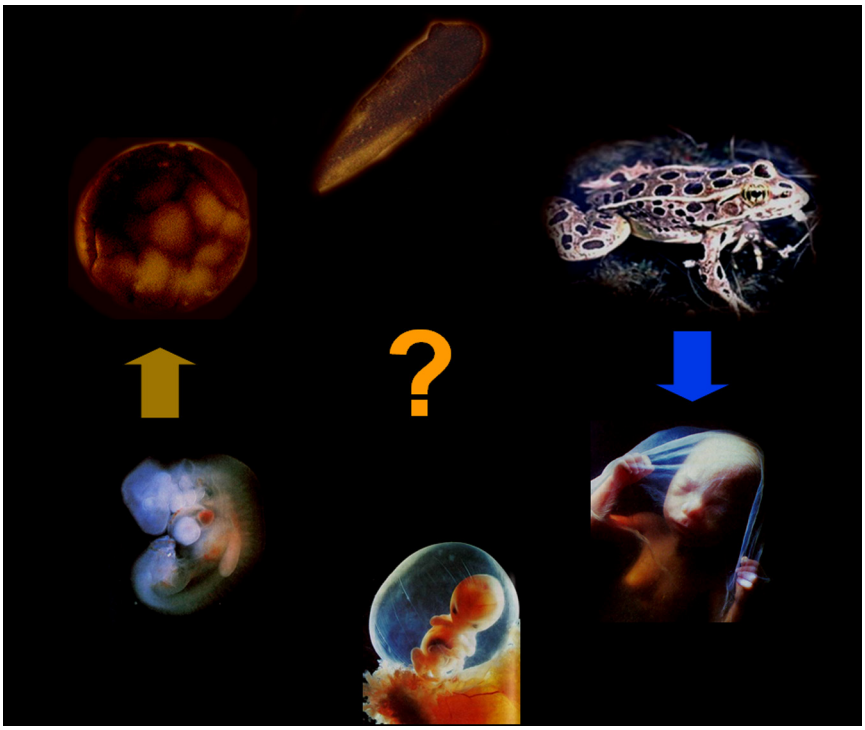
So does atrazine turn on aromatase and increase estrogens in humans? We're not going to worry about egg production in mammals, but what is concerning about aromatase expression and estrogen in mammals is breast cancer and prostate cancer. With regard to prostate cancer, there in an 8.4-fold increase in prostate cancer in men who work in atrazine factories and bag atrazine. There is at least one correlational study, which I didn't publish, that shows women whose well water is contaminated with atrazine are more likely to develop breast cancer than women who live in the same community, but don't drink the well water. (Kettles, M., et al. *Environmental Health Perspectives.* 1997 Nov; 105(11): 1222–1227) Again, it's just a correlational study, but if you look at rats, testosterone goes down when you give them atrazine and estrogen goes up. Syngenta's own studies (1994) show that, if you give rats atrazine, there is an increase in breast cancer and mammary cancer (see Figure 4). The mechanism for prostate and breast cancer is the following. Adrenal cells normalize aromatase and estrogen production to one, but if you give these human cells atrazine they express aromatase and start making estrogen. Like we have shown in fish and amphibians —and just like they've shown in reptiles, just like they've shown in rats— lo and behold, human cells respond the same way. Now there is a mechanism to go along with our experimental evidence in rats, to go along with our correlational evidence for breast cancer.

One of my graduate students has shown that, if you take breast cancer cells and give them atrazine, they start expressing aromatase and start making estrogen. Now here's why that's important. It turns out that aromatase is typically expressed in those cells around breast cancer. Otherwise, think about it —most women get breast cancer after menopause, when estrogen levels are lower than they have ever been in their lives. How can that be? That's because one, breast cancer incidence depends on your lifetime exposure and, two, it depends on this local expression of aromatase. In fact, aromatase expression is critically important in causing that cancer to grow, elevating your own production of estrogen that binds to the receptor and causes the cell to divide.

Produce the Poison, Profit on the Treatment

Who knows what the number one treatment for breast cancer is right now? The chemical called letrozole (Femara®) knocks out aromatase and decreases estrogen so that those cells don't turn into a tumor. How much sense does that make, when the number one contaminate of drinking water, bathing water, ground water, surface

Figure 5



Dr. Hayes' research has led him to believe that human fetal exposure could lead to similar effects observed in frogs. Slide image courtesy Tyrone Hayes, Ph.D.

water, and rain water does exactly the opposite in every animal that's been examined and is associated with breast cancer in humans and promotes and induces breast cancer experimentally in rats.

Here is where I get in trouble. The same company that's given us atrazine since 1958 now makes letrozole. Novartis Oncology offers treatment for cancers. The same company that gives us 80 million pounds of atrazine, an aromatase inducer that promotes breast cancer in rats and that's associated with breast cancer in humans, now gives us letrozole to knock out aromatase, to basically, I would argue, undo what it did.

Effects Across Generations

I would argue that my love and study of this aquatic organism , the frog, has taught me quite a bit about another aquatic organism, the fetus. The things that we're studying in frogs are relevant to the things we are studying in humans. Some might question that, but I would argue that my tadpole trapped in a contaminated pond, is no different than the fetus, trapped in a contaminated amniotic fluid dependent on the same hormones as my frog. Studies now show that before we leave the womb, we are exposed to 300 chemicals, most of which have effects that are not understood.

Here now is the work that changed my life. An EPA lab showed that if you give pregnant rats atrazine, it will cause an abortion. (Cummings, AM., et al. *Toxicological Sciences*. 2000 Nov;58(1):135-43) It causes so much of a hormone imbalance that the rats can't maintain the pregnancy. A second EPA lab showed that, of those rats that don't abort, the sons are born with prostate disease. (Stanko, J., et al. *Reproductive Toxicology.* 2010 Dec;30(4):540-549) A third EPA lab showed that those rats that don't abort the daughters of the exposed mothers are born with poor breast development and essentially their offspring have retarded growth and development because they can't make enough milk. (Rayner, JL., et al. *Toxicology and Applied Pharmacology*. 2004 Feb;195(1):23-34) Here is what changed me profoundly: we're seeing rats affected by atrazine that their grandmothers were exposed to. These are rats that never saw atrazine.

When I think about my little girl, it gives me a whole different vision. We publish in the ivory towers and journals, such as PNAS and *Nature*, that mean so much to our promotion and our academic colleges, but we publish in places that 99.9% of the world doesn't have access to.

I have told you there are birth defects in rats, but there is also a correlation between human birth defects and conception that occurs during peak atrazine. A study published by the Centers for Disease Control and Prevention concluded that maternal exposure to surface water atrazine is associated with fetal gastroschisis, particularly in spring conceptions. (Waller, SA., et al. *American Journal of Obstetrics and Gynocology.* 1010 Mar; 202(3):241)

I was told when I got involved in this, "Don't be an advocate, Tyrone. Let the science speak for itself." For many of us scientists in the ivory tower, we take that attitude because that is how we were taught. When I found that the idea of letting the science speak for itself really meant that my science was being spoken in PNAS, *Nature*, and places that don't reach the public, I knew I had a completely different responsibility. One, as an academic scientist, but two, to make sure that information is available wherever it is needed and whoever would tolerate me for 30 to 40 minutes to talk about it.

I figure I didn't grow up privileged, but I've had the benefit of some really fancy education and I know what's going on. Now I have a different philosophy, "Those who have the privilege to know, have the duty to act." I wish more of my colleagues would follow that philosophy.

Please consider supporting Dr. Hayes' continued research by keeping his lab open. Go to www.beyondpesticides.org/fundscience to pledge your support.