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Statement to SBX 2 Nitrates Expert Panel

Bud Hoekstra, BerryBLest Farm, 9 May 2014

Part 1

Introduction: The farming magazine SUCCESSFUL FARMING featured nitrate pollution in the Chesapeake Bay in its mid-February issue. Among the stories and anecdotes that unfolded in the attempts of farmers and law-makers to whip groundwater pollution was the true tale of Steve Berger, a farmer in Wellman, Iowa, who "remembers when the first yield monitors came out." During harvest time, his monitor registered major yield bumps "where fencerows used to run through my fields." Think about that. Yield bumps are every farmer's dream. Conventional farmers want their fields to perform like fencerows, and Organic farmers think their fields do – if they play by nature's rules and observe nature's limits. Fencerows have no upkeep. The soil is not plowed; not fertilized, not seeded, perhaps sprayed by drift. Yet in the giant compendium of human knowledge about agriculture, seldom does a tended field outperform an untended fencerow.

In the article by Dan Looker and Kacey Birchmier "A Tale of Two Ways to Clean Water," the authors noted something of an explanation for the fencerow productivity. They wrote, "Currently, where he has fencerows, the organic matter is 6%, but the organic matter in his field is closer to 3%." Walla! Organic matter is an underground reservoir of plant nutrients, and the bigger the reservoir, the bigger the yield. Conventional farmers pump nutrients into a shallow reservoir, inasmuch as the organic farmers build deeper reservoirs. And this comes as no surprise: Organic farmers make soil, conventional farmers use fertility up and replace it by other means.

Law-makers in Maryland cracked down on nitrate pollution and the state has some of the toughest rules in the nation. In Maryland, you even need a license to apply fertilizer. SUCCESSFUL FARMING [same issue, page 14, "Lessons from the Bay"] gives an overview of the state's nitrate policy: "The unintended consequences are numerous, like the conventional dairy farm of Red Fry, who also has an organic grain farm. He's planning to convert 500 acres to conventional corn, because he'll have to switch from manure to chemical fertilizer on that crop ground." Nitrate regulation slams Organic farmers!

The irony makes me rigid with disbelief. The National Organic Program [7 CFR 205] defines organic agriculture as "…practices [that] maintain or improve … water quality." And "Lessons from the Bay" deliver the first lesson: "The requirements are essentially university-based best-management practices." Here we are with a revolting contradiction: Organic farming is a set of practices that protect water quality, but the new rules that protect water quality disfavor Organic methods.. Maryland's laws purport to quell nitrate pollution in groundwater and the Bay, and

MANAGING

YOUR FAR

By Dan Looker, Business Editor

LESSONS FROM THE BAY

THE CHESAPEAKE IS UNIQUE, BUT CLEANUP EFFORTS SHOW OPPORTUNITIES ELSEWHERE.

Efforts to clean the Chesapeake Bay hold lessons for all of us, especially those who farm in the much larger Mississippi River Basin. After nearly a week of visiting farmers in Maryland and Delaware, I came away with three things you need to know.

First, it really is as draconian as you may have heard. The requirements are essentially university-based best-management practices. Yet, as you'd expect, I met no one who really likes the threat of an audit or fines, or the paperwork that goes with having an annual nutrient-management plan. It's worse than my article on Maryland in this issue makes it seem (see p. 44), just because I didn't have room to describe the proposed fees on irrigation water and other ideas coming out of Maryland's mostly urban legislature. New rules that will ban P in soils with high levels will make life even tougher for poultry and dairy farms that already have a challenge finding places to take it. The unintended consequences are numerous, like the conventional dairy farm of Ed Fry, who also has an organic grain farm. He's planning to convert 500 acres to conventional corn because he'll have to switch from manure to chemical fertilizer on that crop ground.

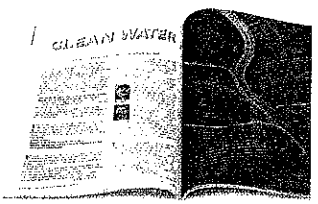
Yet, Maryland and Delaware farmers have learned some things that could help you survive the new normal of tighter margins in crop production. Their second lesson is that nutrient-management plans, if they're farmer-friendly and flexible enough, can save you money.

As my story points out, after a decade of regulation, Maryland farmers who take a two-hour training session to get a nutrient voucher every three years believe the training (and the nutrient-management plans) have saved them an average of $7.49 an acre. That's based on a confidential, scientific sample conducted for University of Maryland Extension by an independent polling firm.

A fourth of those farmers say they're saving more than $11 an acre. That's close to a tenth of current University of Illinois estimates of the cost for fertilizer this year in southern Illinois ($125 an acre). Fertilizer is about a fourth of Corn Belt nonland costs.

One caveat is that most farmers surveyed weren't certain of the economic benefits of the training. I don't know if that represe confusion from the way t question was asked or wh it represents inadequate r cords. But the study certa backs up farmers who sai they've increased profits v fertilizer management. [I was in last May's *Journal o the National Association of County Agricultural Agent.* To find it online, search for "Maryland Nutrient Management Training: Impacts Assessment."]

In neighboring Delaw helping farmers become more efficient has alway: been a top goal of the sta Nutrient Management Commission, says Dave



For more on nutrient management, s stories starting on page 44.

Photography: Dan Looker

Organic farming apply the same BMP's to achieve the same end. But the Maryland laws undermine the clean water practices of Organic farmers, so it seems.

Thus, when I read CDFA's ***Nitrogen Tracking and Reporting Task Force: FINAL REPORT, December 2013***, I became so alarmed, and its that alarm that brings me here today. Please hear me out. Page 9 of the SBX 2 1 final report names the Maryland Nutrient Reporting Program as one that's relevant to California.

Everyone knows the heinous tale of the Oklahoma City bombing, Timothy McVeigh and Terry Nichols and the entertwined story of their story of using as an explosive. The entertwinement goes back a hundred years when Germany cut the U.S. off from potash in 1912, before world war I. The Interior department hired potash inspectors who combed the West looking for potash ores. The story ends with U.S. Potash of New Mexico and the addition of Calsbad Caverns as a unit to the national park system. Fritz Haber, the father of modern agriculture, invented his process for making nitrates from air – and he commercialized another of his ideas, the first pesticide used in food handling and storage – a product that later emanated from the showers at Auschwitz and took Haber's relatives' lives. The War established these industries, and after the war, the supply of minerals exceeded the demand for them. The industries propped up the wartime production levels with a spin-off technology of fertilizer. It was this aftermath era that gave rise to Steiner and Lady Balfour who championed the Organic revolution that we witness today.

Europe had the same nitrate problems as America, and some European nations subsidized Organic farming to curb the taints of agricultural pollution and make farms more economically resilient. Between roughly 1990 and year 2000, Austria went from 1200 certified Organic farms to 19,000. The report appears in Conservation Ecology "Organic Farming and Social-Ecological Resilience: the Alpine Valleys of Solktaler, Austria" by Milestad and Hadatsch. Organic farming does not allow the use of synthetic fertilizers, and the shrinking markets of Europe scared the industry in America which took steps to preserve its market share. No less frightening to the captains of the fertilizer industry were the 2008 Gulf Hypoxia Action Plan that, according to SUCCESSFUL FARMING [same issue, page 44], "would mean a total reduction of nitrogen … loads by 45%." Industry's sales would be doomed to plummet almost in half.

With the fertilizer industry a-fighting to maintain its market share, how will California's water legislation turn out? Will it be Organic-friendly or not?

Stacking Science: Fertilizer is all about plant nutrition, and we all want farms to be sustainable. We all want plants to grow. Soil today is soil tomorrow, to put it succinctly. The fencerow conundrum still visits its ironies on today's farms – whether they be conventional or Organic. Neither methods have been perfected to grow crops without polluting.

In the new Springer Journal CBTA, Chemical and Biological Technologies in Agriculture, Alessandro Piccolo writes, "Obvious issues are related to plant-soil-microbe interactions in order

to improve knowledge on plant nutrition that in some respects is still anchored in Liebig's traditional concepts of inorganic **nitrogen** uptake, while the complex world of chemical, biochemical and microbial interactions involved in the '*organic*' nutrition of plants is still to be unraveled." In other words, we have some science on how to dump nutrients into a crop field, but science falls short of orchestrating the "fencerow" soil biota to the same – Organic methods remain a recondite art.

Liebig shed a light on farming, but his light was only a dim oil lamp. Quoting CBTA's first issue, "structure-activity relationships between plant-rhizospehere-microbe and biomolecules, as well as advanced molecular releasing systems, will be of primary importance for a future sustainable agriculture." We need a greater light spotlighting the biological life of the living soil.

What does this mean? In brief, nitrates are the tie that binds all methods of food production, and all nitrates go to one or all of five sinks: the air, the groundwater, the soil humic storage, the plant uptake or surface water runoff. The aim of farming and regulation is to keep groundwater and surface watercourses free of nitrates. By a process of elimination, after eliminating the two sinks, nitrates must be taken up by plants, dispatched to the air or earmarked into the soil.

Therefore, we turn to science for sink management and direction. We may well ask which system of farming works better at nitrate control? Organic or conventional? We can also ask how we may perfect either system to curb the run-away nitrate pollution embedded in the green revolutions of food production. What are the practices that keep nitrates out of groundwater? What are those cherished BMP's?

Here's where the expert panel has to turn on their built-in, shock-proof shit-detectors to read and absorb hundreds of pages of science aimed at protecting, preserving and perpetuating the living soil or – otherwise – protecting, preserving and perpetuating fertilizer markets. Administrative law books [example: UC-Davis uses Emanuel law outlines, Jack Bergman's Administrative Law" "public interest theory" versus "public choice theory"] remark on the dichotomy of our laws, often commencing with a noble purpose in mind to serve the public good all the while being compromised or digressed into something of an aegis shielding markets. It's in the nature of the decisions of a political animal that corporate and public interests collide. It's in the public interest to get nitrate fertilizer out of the groundwater, but the fertilizer industry wants to keep their market share and sales.

Ruckelshaus, when he headed up the Reagan EPA, quipped that science was like any animal, put a ring through its nose [a funding ring] and you can lead it anywhere. Science shapes policy, but policy can shape the direction of science! Also, in science, truth is more a sense of direction than a correct answer. Hence, scientific literature is filled with studies that seemingly contradict. Studies are mini-steps in giant step of political action. Controversy is always good – it sharpens the insight; conflict and manipulation are bad, because the bad science that results misleads

society, policy and legislation. We need good science, controversy can get us there, manipulation of science to fix policy derails its integrity.

To make the point, we will sample some of the contradictions of nitrate science, some of it genuine and some of it spurious. Some of the science elucidates the nature of nitrate pollution; some of it shores up the position of the fertilizer industry or the organizations that promote Organic methods. To be open-minded, fair and expert, the panel needs their built-in, shock-proof, crap-detectors to discern the truth.

Get out your score cards!

Here's a scientific study that finds intensive conventional agriculture less likely to pollute groundwater than organic production. Dahan et al. [Hydrol. Earth Syst. 18:333-341, 2014] completed a two year study of greenhouse pollution of groundwater. Their title "Nitrate leaching from intensive and organic farms to groundwater" encapsulates their study, though the organic farm had a 69-year history of intensive agriculture before putting up a greenhouse. Their conclusion was this: "Similar intensive agriculture that implemented liquid fertilizer through drip irrigation, as commonly practiced in conventional agriculture, resulted in much lower rates of pollution [nitrate] of the vadose zone and groundwater."

Conventional farms outperform organic farms in nitrate leaching, their research demonstrates.

Here's another study which concluded no differences in nitrate leaching!

Kirchmann and Bergstrom's "Do organic farming practices reduce nitrate leaching" appeared in the journal "Communications in Soil Science and Plant Analysis [32(7-8), 2001]" and concluded "If the different input intensities of nitrogen between organic and conventional systems were taken into account and corrected for, no differences in leaching losses between systems were found ... reduction of nitrate leaching is not a question of organic and conventional farming but rather of introduction and use of appropriate counter-measures." I take their term "appropriate counter-measures" to mean BMP's, and according to 7 CFR 205.215, organic farming a set of BMP's that "maintain or improve ... water quality." The National Organic Program has built-in, required BMP's.

Though confused about it, I'd call this one a draw on my scorecard. The next study favors Organic methods and suggests that nitrate leaching is less with Organic farming.

Pacini, Wassink, Giesen, Vazzana & Huime presented their study in the journal Agriculture, Ecosystems and Environment 95(1):273-288, 2003, titled, "Evaluation of Sustainability of organic, integrated and conventional farming systems: a farm and field-scale analysis." Their conclusion was this: 'The OFSs [Organic farming systems] perform better ... with respect to nitrogen losses."

Organic scores one here. But here's another study that presents quite whopping, dramatic results in favor of Organic methods of production.

In a UK-published book, Haas, Guido et al. compiled the chapter "Nitrate leaching: comparing conventional, integrated and organic agricultural production systems" that appeared in the book AGRICULTURAL EFFECTS ON GROUND AND SURFACE WATER edited by Steenvoorden, Joop, Claessen, Franz, Willems and Jaap. The authors of the study were associated with the International Association of Hydrological Science or the Institute of Organic Agriculture, and their conclusion was profound: "...converting to organic farming reducing leaching losses of nitrogen by more than 50%."

This is a coup for Organic farming, a home-run hit, because organic farming performs as it was intended to perform. My scorecard may record a triple score for this coup! And this study is a slap in the face of the fertilizer industry whose rise in the market parallels the decline of groundwater in the United States due to nitrates. But the jury is still out, and the science is contradictory, as evidenced by this selection of peer-reviewed studies.

I found these studies – all four in full PDF – on the Internet, but one index word will not suffice to get you to all four. When I began exploring the scientific literature on nitrates, I discovered a plethora of related terms that seldom overlapped and each term brought up related concepts and practices. Why? No one discipline studies nitrate movement and pollution of groundwater. Forestry is one discipline, agronomy is another. There is hydrology, microbiology, the science of adaptive management – even the EPA has a website that evaluated the potential of BMP's to work effectively to control nitrate pollution. And then there's engineering and environmental toxicology too. So here are some of the diverse terms covering the same area and subject.

Nitrogen conservation

Precision agriculture

Nitrogen budget

Dissipation half-life

Field dissipation

Hydraulic residence

Soil nutrient retention

Nitrate sorption

Nitrate leaching

Bridging

"Hydraulic residence" is what engineers use, and "bridging" describes the role of water in holding nutrients to humic substances in the soil. The EPA published a textbook for regulators titled MANAGEMENT MEASURES FOR THE CONTROL OF NONPOINT POLLUTION FROM AGRICULTURE which contains information on surface and groundwater pollution with nitrates. All have their own language for nitrates. The nitrate issue is interdisciplinary.

The EPA uses adaptive management as its regulatory principle: do-measure-adjust. Farmers face standards of water quality or choose their own management measures [goals] and they meet those standards or measures with practices that prevent exceedances of mcl's or TDML's or their own management measures. The practices are BMP's, and it takes a suite of BMP's to control and manage nitrates. Not one BMP, but a panoply. Organic agriculture tends to be a panoply by law.

Organic farmers are certified by USDA-accredited certifier, and the certifier has the farmer prepare an OSP, organic system plan, that lays out the BMP's that the Organic farmer will use. The certifier comes and checks the farmer's records to see that the farmer has complied with the provisions of his own OSP.

Some of the provisions of the OSP are mandated by law. For example, the BMP of cover crops is required by 7 CFR 205, and organic farmers must include it in their OSP.

I looked at the BMP's that Organic farmers typically use to farm, and four of them stand out as affecting nitrate sinks:

Cover crops

Residue mulching

Composting

Crop rotation

These practices or BMP's nurture the soil microorganisms that can lead to less leaching

Then I did a meta-analysis of the Central Valley Regional Water Quality Control Board's preferences to see if there was bias or prejudice – to determine if the fertilizer industry was protecting its markets through the regulations or if the game board was a level playing field.

Engineers are minimalists – they rely on minimization technologies to control pollution. The Clean Water Act of 1972 started with point-source ["outfall pipe"] pollution, and the way to reduce pollution was to re-engineer the industrial processes to minimize. Minimization was the goal. Occasionally, other devices served to meet the regulatory requirements – Environmentalists scoffed at some of these methods - "the solution to pollution is dilution," they jeered with reason. Sometimes, whole new methods of manufacture were devised and deployed. The sugar industry learned to recrystal sugars from their waste water to minimize their pollution.

The staff of CVRWQCB are chemical engineers, and I expected them to push minimization technologies in their regulations.

My meta-analysis:

The fertilizer industry wants to protect its market for sales and preserve its corporate revenues. If, as Europe has done, regulation favored Organic methods, organic agriculture would receive a big push – and since organic farmers do not use synthetic fertilizers, the industry would lose out in the marketplace. Industry has a push of its own, so to speak, and that push is the choice of sinks: plant uptake. The idea behind it is called ***precision agriculture***, and fertilizer is doled out to meet the expected crop uptake. Farmers in high-risk zones for groundwater contamination are limited to applying 1.4 times the nitrogen needed by the crop for the expected yield, by the new CVRWQCB rules. This presumes that farmers were prodigal and purchased excess fertilizer for fields so that cutting back will protect the groundwater.

Now then, the meta-analysis: what I did was to compare the list of BMP's that were acceptable to CVRWQB to see if those BMP's fell into a category of minimization technology, uptake efficiency [precision agriculture] or genuine organic methods as previously listed.

I found a CVRWQCB bias for the fertilizer industry and for precision agriculture.

For example, Pamela Creedon of the CVRWQB approved a proposed farm evaluation template for the Westside San Joaquin River Watershed Coalition and page 2 of the template approved on 28 March 2014 listed "Nitrogen Management Methods."

*"Cover crops" was the first item on the list, and cover crops are an uptake mechanism as well as an Organic BMP [see NRCS conservation practice standard] This BMP falls into two categories: Organic farmers use cover crops to enrich the soil, a "nutrient "sink;" and conventional farms use it as plant uptake, also a holding "sink" for nitrates.

*"Split fertilizer application" was the next item, and the item falls into the category of precision agriculture.

*Soil testing is universal, required of organic farmers, optional in conventional agriculture. Organic farmers use it to show that the soil quality is maintained or improved. Conventional farmers manage nutrients through precision agriculture. I decided that precision agriculture was what was meant here, to meet the "1.4 X" rule for groundwater vulnerability areas.

Likewise, *tissue/petiole testing supplements precision agriculture to minimize nutrient application to what the plant needs and will take up to produce an expected yield.

*Variable rate applications using GPS is indisputably precision agriculture.

*Foliar N application is minimization technology – nitrate is not applied to the soil.

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Abstract

Humic substances (HS) have been widely recognized as a plant growth promoter mainly by changes on root architecture and growth dynamics, which result in increased root size, branching and/or greater density of root hair with larger surface area. Stimulation of the H^+-ATPase activity in cell membrane suggests that modifications brought about by HS are not only restricted to root structure, but are also extended to the major biochemical pathways since the driving force for most nutrient uptake is the electrochemical gradient across the plasma membrane. Changes on root exudation profile, as well as primary and secondary metabolism were also observed, though strongly dependent on environment conditions, type of plant and its ontogeny. Proteomics and genomic approaches with diverse plant species subjected to HS treatment had often shown controversial patterns of protein and gene expression. This is a clear indication that HS effects of plants are complex and involve non-linear, cross-interrelated and dynamic processes that need be treated with an interdisciplinary view. Being the humic associations recalcitrant to microbiological attack, their use as vehicle to introduce beneficial selected microorganisms to crops has been proposed. This represents a perspective for a sort of new biofertilizer designed for a sustainable agriculture, whereby plants treated with HS become more susceptible to interact with bioinoculants, while HS may concomitantly modify the structure/activity of the microbial community in the rhizosphere compartment. An enhanced knowledge of the effects on plants

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*Irrigation Water N testing is used to compute the nitrate rate of application in precision agriculture.

*Fertigation is a technique of precision agriculture.

What was missing are very definite, "university-based" BMP's that are used in organic agriculture. Here are examples of Organic practices that increase the N-retention capacity of the living soil.

**Buffer strips – buffer zones have conservation covers [see NRCS] and the Leopold Center for Sustainable Agriculture, for one example, showed that nitrate losses to streams can be reduced 90% by a two-meter buffer. Streambeds serve as conduits whereby nitrate-laden water seeps into the ground and contaminates groundwater. This organic BMP is not on the "Westside" list.

**Residue mulching, and the more general "mulching" with organic materials builds humus in the soil that retains water and thus retains nitrates that would escape the root zone and taint the aquifer water. Mulching was not found on the list; residue management was not found on the list. Both of these are Organic BMP's that control nitrate leaching. In fact, it is well known that chip mulches and sawdust feed mycelium in the soil, which in turn creates glomalin, that together with humic substances provide a slow release mechanism for nitrates. Too much woody biomass in the soil can lock up nitrates so that tomatoes do not turn red in the heat of the summer growing season!

**Composting restores and elevates humic substances that serve to retain nitrates and other nutrients that typically leach. The biggest difference in soil quality between farms that are organic and farms that are conventional is humus – and soil organic matter [SOM]. An agronomist at Texas A & M presents a startling picture of U.S. soils. He estimates the overall SOM to be 2% because the plow destroys SOM. He attests to 28% to be the desired percentage of organic matter in the soil for good agriculture. The Organic practice of composting is omitted from the CVRWQCB-approved "Westside" list.

**Crop rotations are used for weed control and the spin-off effect is a healthier soil biota. Healthier soil lends itself to stabilizing the nutrients in place and storing them in the root zone. Humus, glomalin, the saccharides that paste together aggregations of soil particles retain nutrients and prolong their residence time in the root zone. Crop rotation does not appear on the list!

When we find things like this list of Nitrogen Management BMP's and see that they overwhelmingly favor precision agriculture, we know that the Water Board caters to the fertilizer industry and slights Organic growers. I'm beginning to tremble as I see the Maryland NITROGEN TRACKING AND REPORTING TASK FORCE over again!

The irony is that not all BMP's are assured of the effects they produce. Environmentalist decry the cataclysmic loss of worms, their populations devastated by two centuries of the plow. Worms produce humic matter – "worm castings" that retain nutrients in the root zone. Rhode Island researchers disagree, and research is underway to show that the tunneling done by these creatures shunts water and its load of dissolved nitrates past the root zone and into the groundwater. The idea, of course, would be a vermicide that kills these pests so that their tunneling does not produce a loss of nitrogen that spoil groundwater.

Organic farming emphasizes soil makeup – and soil is a sink for nitrates. Nitrates are stored in the sink and during the growing season when plants need nitrogen, soil biota get their act together and release it to the plant. When the season ends, the microbes slow their work down, as demand for nitrates from the plants stops. In theory, groundwater stays clean, because the living soil delivers nitrates as needed.

Conventional farmers are beholden to the fertilizer industry and precision agriculture. These farmers can apply nitrates up to 40% in excess of what the crop needs to assure the expected yield.

The CVRWQCB water board has conspicuously favored "precision agriculture" (the mark of conventional farming) over Organic methods in its approval of the template of the Westside San Joaquin River Watershed Coalition. This is what the fertilizer industry wants – to halt the spread of Organic methods that don't use their products.

Summary picture:

The science in support of Organic agriculture has a consuming interest in the biological life of the living soil, how the microbes interact with nutrients to manage nutrient cycles within the dimension of the rhizosphere. Organic farming research focuses on humic substances which act as stimulants to root growth. Studies have shown a thickening of roots in humic soils compared to the same crop plant in non-humic soil, roughly speaking. Glomalin has been heralded as a carbon reservoir, and glomalin is a "hiding place for a third of the world's stored carbon." [Comis, D. AGRICULTURE RESEARCH volume 4, 2002] The Rodale Institute has offered farmers a new parameter of soil testing – the volume of microbial activity in their garden soil. The plow was a destructive invention, its use resulting in the annihilation ageliad and anecic worms and the banishment of humus from our soils. The theory behind Organic practice is the restoration of the soil – not just the salting of the missing or lost nutrients, but the resurrection of the abundant life and its natural cycles in the soil that are a part of the life-support system of the planet.. Researchers in Organic look at "bridging" – the ability of organic molecule of humus to be hydrophilic and hold onto water and keep it resident in the soils. Bridging also includes the ability of the stabilized water molecule to hold onto mineral nutrients and metals. One Chinese study suggested that humus could capture cadmium, a taint of rock phosphate fertilizer, and prevent its uptake by the plant and into the stuff of our food.

People a 100 years hence will probably look back on the Organic revolution in a manner not unlike that of medicine. 200 years ago, patients were given decoctions of Foxglove leaves, a cure for CHF or "dropsy" and it worked. This was plant-based medicine, but it had its shortcomings. The digitalis found in Foxglove plant occurred in varying quantities, and an exact safe dose of it could not be administered through a decoction of the leaves. Patients died from overdoses until chemists isolated the active ingredient digitalis, and meted it out exactly. Now digitalis is made by mineral-based chemistry, and plants are not harvested for it. But more and more plants find uses in modern medicine, and the peak of mineral-based medicine passed about 1900. ***Mineral-based agriculture*** still thrives on farms and fields, but the store of human knowledge is pushing us toward an ecological understanding of farming. If we strip the ecosystem from the land, we have to replace the lost ecosystem services. Fertilizer NPK "services" the losses caused by the plow. As history unfolds, we will see either humus made from minerals in a factory or we will see the Haber process become obsolete in agriculture. The driving force or impetus behind the change will be the need for clean water – it is more cost-effective to keep water clean than to purify it once it is contaminated.

Conventional farmers tend to think of NPK fertilizers as plant nutrition and yield-maximizers. Organic farmers see it as an ecological correction, when the ecology is squeezed out of farming.

The jarring truth about water resources is that in the span of 200 years, the U.S. went from "pioneers kneeling at natural springs to cup their hands and slate their thirst" to bans and restrictions on well water because of nitrate use and photos of firemen hosing down rivers with water to put out the burning pollution. What a terrible irony: when rivers catch fire, hose them down! Organic farming began as a movement to protect, preserve and perpetuate the quality and quantity of living soil and its store of nutrients. Soil is not a mere magazine of plant nutrition, it is a living, biological system that cycles nutrients within itself. Conventional agriculture, especially the plow, has spoiled the soil and used its fertility up. Industrial-ag hasn't even lived up to the "fencerow" promise of production. In the future, it may be possible that precision agriculture will curb runaway nitrate pollution, but in the meantime, the SBX 2 expert panel should advise law-makers to give Organic a fair chance to prove its ultimate worth of sustaining soil and water while protecting the food chain from poisons.

Part 2

The main rallying point for nitrates is the mcl of 10 milligrams per liter Nitrate-nitrogen, or the equivalent, 45 mg/L of nitrate ion, based on observations of infants with methemoglobinemia in 1948.

Deoxygenated blood, anoxemia, has diagnostic blood color, depending on the agent causing it.

Condition	diagnostic color
Anoxia	cyanotic "gray-blue"

Carboxyhemoglobinemia	cherry red
Methemoglobinemia	chocolate brown/blue
Sulfhemoglobinemia	greenish blue

Infant bodies lack an enzyme called cytochrome reductase or methemoglobin reductase that pries off the meth group to return hemoglobin to its normal oxygen-carrying state. Some people are born without the genes that code for this reductase, and are at higher risk than a normal adult of methemoglobinemia. Most people have 1-2% methemoglobin in their blood, but the reductase keeps the level at bay. Without the reductase, levels shoot up to 40-50% which is a critically high level and prone to anoxic mortality.

In 1948 a pair of doctors dosed four infants with nitrates to induce methemoglobinemia. Above 10 mg/L doses produced the "blue baby syndrome" and 10 mg/L became the safe level supported by the medical literature. But the safe level is not without controversy.

Lorna Fewtrell, writing in the respected journal EHP, Environmental Health Perspectives, took issue with the U.S. and World Health Organization's 10 mg/L guideline. [EHP 112(14):1371-1374 Oct 2004]. Her article was titled "Drinking-Water Nitrate, Methemoglobinemia: A Global Burden of Disease" and urged the WHO to relax its standard to 50mg/L.

In a contrasting EHP article in year 2000, another author asks, "Does the risk of childhood diabetes require revision of the guideline values for nitrate?" The EWG, Environmental Working Group, gave its white paper in February of 1996 "Pouring it on: Health Effects of Nitrate Exposure" which urged a toughening of the guideline standard - lowering it to 3 mg/L from the EPA standard of 10 mg/L.

The WHO has a multiple guidelines for nitrates – nitrates tend to reduce to nitrites by the action of bacteria in the mouth and the gut. Up to 20% nitrates become nitrites in the mouth, it has been estimated. WHO sets a guideline of 11 mg/L for pure nitrate-nitrogen and 0.9 mg/L for pure nitrite-nitrogen in bottle-fed infants. However, the WHO guideline is 1.0 mg/L nitrogen in mixtures of nitrates and nitrites.

To quote EWG, "The current EPA standard does not adequately protect public health." Some professionals would go a step farther and enforce the provisions for private wells. Carl Johnson MD, MPH and Burton Kross PhD, PE, wrote "Continuing Importance of nitrate contamination of groundwater and wells in rural areas." [American Journal of Industrial medicine 18(4):449-456, 1990] To quote them, "The contamination of groundwater and rural drinking water supplies ... is a potential hazard throughout the world. Infant illness and death from nitrate-induced methemoglobinemia is probably often misdiagnosed, perhaps as sudden death syndrome, and certainly contributed to the national infant death rate statistics. A 1950 report listed 144 cases of infant methemoglobinemia with 14 deaths in one 13-month period in Minnesota. Infant deaths

resulting from this preventable, treatable intoxication were still occurring as recently as 1986 in South Dakota. In this state, about 39% of dug or bored wells were unsafe due to high nitrate content compared with 22% of drilled wells and 16% of driven wells. Properly constructed wells more than 30 m deep are more likely to be safe. Groundwater concentrations of nitrate maybe unsafe for consumption, and standards are needed to regulate such contamination. Such standards could serve as guidelines and be enforceable in the case of water systems dependent on wells."

The first medical article appeared in JAMA [129:112-116] in 1945: "Cyanosis in infants caused by nitrates in well water."

Adults also present at hospitals with methehoglobinemia, usually due to ingestion of drugs or other chemicals. In one case, a suicidal hair dresser took hydroxylamine sulfate and suffered both methemoglobinemia and sulfhemoglobinemia together. Methylene blue cures methemoglobinemia, sulfhemoglobinemia is incurable. A commonly abused drug, organic forms of nitrite called poppers, can induce methemoglobinemia. These drugs are legal in many countries and can be purchased on the Internet.

Nitrates are found in many household products, including toothpaste, and chances are you had a dose of nitrate this morning when you brushed your teeth. The amount of nitrate in water pales in comparison to what is in our food.

Whereas nitrate is just one chemical and as we argue about the safety standard of that one chemical, we ignore the bigger problem of mixtures.

Standards are developed for individual chemicals, and these standards do not take into account the gamut of exposure to cocktails of chemicals. Cocktails are a reality of pollution. As the Silent Spring institute put it, "We are exposed to complex mixtures, but scientists study one chemical at a time."

Indeed, 2, 3, a dozen, 10,000 chemicals can be additive in effect, multiplicative [synergistic] and subtractive [antagonistic] in their impact after exposure. Often, two chemicals act in concert as though with the effect of one, and inasmuch as the levels of each are individually safe, the combination of the two has an effect on health that is unsafe.

The EPA does not regulate mixtures, but increasingly, science shows that mixtures are responsible for leading health effects and diseases. As the Cape Cod Times put it, "Water concerns go beyond nitrates."

A further irony is the hormone-like activity of some chemicals in a mixture. These chemicals can exert an effect at low concentrations that are deemed safe. The Silent Spring Institute drew this picture of what's come to be called EDC's, or endocrine-disrupting contaminants. "Endocrine disruptors can show effects at lower doses that are not apparent at higher doses."

Scientists sometimes refer to ***non-monotonic*** nature of these chemicals that exert an effect at low doses but not at high doses – defying regulatory principles and confusing science.

Once upon a time the world of toxic substances was simple: above a threshold dose, a poison was a poison; below that threshold, the substance was safe. "Dose makes the poison" was the old adage. Selenium is a good example. We need a trace amount of selenium in our body for healthy blood; at high doses, selenium can interfere with bone or nail formation, among things. The EPA sloughed off the threshold model for the dose-response model, meaning that the higher a dose, the greater its effect or risk. Even when the Ruckelshaus EPA took on the new dose/response model, scientists were speculating on carcinogenesis – cancer – which manifests as a multi-stage model. Now we face a non-monotonic model presented by hormone disruptors. The challenge is there, the regulation is not – not yet.

Nitrate is a EDC, an endocrine disrupting compound. A good estimate of its endocrine-disrupting capabilities can be found in an article by Louis J Guillette and Thea M Edwards, enclosed in the attachments to my statement. [Integr. Comp. Bio. 45: 19-27, 2005] the title is "Is Nitrate an Ecologiclaly Relevant Endocrine Disruptor in Vertebrates?"

Even more enlightening is Heather J Hamlin's dissertation, 2007, University of Florida titled, "Nitrate as an endocrine-disrupting contaminant in captive Siberian Sturgeon, Acipenser baeri."

The results! **"1.5 mg/L caused higher concentrations of estrogens and androgens in the blood." She added, "nitrate does alter the associated stress response..."**

In another scientific peer-reviewed article, James Jaeger, Ian Carlson and Warren Porter explained, "Endocrine, immune and behavior changes occurred due to doses of mixtures, but rarely due to single compounds at the same concentrations." Their article which appeared in the journal TOXICOLOGY AND INDUSTRIAL HEALTH was titled "Endocrine, immune and behavioral effects of aldicarb (carbamate), atrazine (triazine) **and nitrate (fertilizer) mixtures at groundwater concentrations.."**

Study after study implicates mixture-loading. As an endocrine disruptor, NITRATES are linked to birth defects – see the attached study.

A Wisconsin study looked at nitrate in well water: "Well water in karst region of northwestern Wisconsin contains estrogenic factors, nitrate and bacteria." [Water Environ. Res. 85(4):318-326, 2013, Baver, Wingert & Zorn]. To quote the peer-reviewed article, "levels of estrogenicity ... approached a threshold concentration..."

Heather Hamlin, while telling the industry that nitrate is matter of management, also cautions, "In the realm of endocrine disruption, the level of nitrate that is likely to cause concern is unknown."

At one point, the EPA considered a standard of 5 ng/L estradiol equivalency.

Other studies confirm this viewpoint: Nicholas Cipolett "Nitrate alters steroidogenesis in fathead minnows." Or Edwards and others, "Southern Toad Tadpoles exposed to various concentrations of nitrates responded differently depending on the the source of fresh water." One can infer that unidentified estrogenic contaminants in the various fresh waters interacted with the nitrate exposure

For humans, the implications are much the same. EDC's harm people, and the collective effect of many estrogenic or androgenic chemicals can harm. Heather B Patisaul and Heather B Adewale's peer-reviewed article is attached and provides an excellent overview and history: "Long-term effects of environmental endocrine-disruptors on reproductive physiology and behavior." The article appears in FRONTIERS IN BEHAVIORAL NEUROSCIENCE 3:10-2009. Likewise, the Endocrine Society has published their scientific statement on EDC's that appears in ENDOCRINE REVIEWS 30(4): 293-32 , 2009

Inasmuch as nitrates pose a methemoglobinemia threat to infants, nitrates pose a greater threat in the realm of endocrine disruption. This science is new, cutting-edge science and the lack of regulation is not cause to omit it from any task force report.

Bud Hoekstra

8 May 2014

Toxicol Ind Health. 1999 Jan-Mar;15(1-2):133-50.

Endocrine, immune, and behavioral effects of aldicarb (carbamate), atrazine (triazine) and nitrate (fertilizer) mixtures at groundwater concentrations.

Porter WP[1], Jaeger JW, Carlson IH.

Author information

Abstract

This paper describes the results of 5 years of research on interactive effects of mixtures of aldicarb, atrazine, and nitrate on endocrine, immune, and nervous system function. The concentrations of chemicals used were the same order of magnitude as current maximum contaminant levels (MCLs) for all three compounds. Such levels occur in groundwater across the United States. Dosing was through voluntary consumption of drinking water. We used fractional and full factorial designs with center replicates to determine multifactor effects. We used chronic doses in experiments that varied in duration from 22 to 103 days. We tested for changes in thyroid hormone levels, ability to make antibodies to foreign proteins, and aggression in wild deer mice, Peromyscus maniculatus, and white outbred Swiss Webster mice, Mus musculus, ND4 strain. Endocrine, immune, and behavior changes occurred due to doses of mixtures, but rarely due to single compounds at the same concentrations. Immune assay data suggest the possibility of seasonal effects at low doses. We present a multiple-level model to help interpret the data in the context of human health and biological conservation concerns. We discuss six testing deficiencies of currently registered pesticides, and suggest areas of human health concerns if present trends in pesticide use continue.

Curr Microbiol. 2010 Jan;60(1):42-6. doi: 10.1007/s00284-009-9499-3. Epub 2009 Sep 16.

Biological remediation of groundwater containing both nitrate and atrazine.

Hunter WJ[1], Shaner DL.

Author information

Abstract

Due to its high usage, mobility, and recalcitrant nature, atrazine is a common groundwater contaminant. Moreover, groundwaters that are contaminated with atrazine often contain nitrate as well. Nitrate interferes with the biological degradation of atrazine and makes it more difficult to use in situ biological methods to remediate atrazine contaminated groundwater. To solve this problem we used two reactors in sequence as models of in situ biobarriers; the first was a vegetable-oil-based denitrifying biobarrier and the second an aerobic reactor that oxygenated the denitrifying reactor's effluent. The reactors were inoculated with an atrazine-degrading microbial consortium and supplied with water containing 5 mg l(-1) nitrate-N and 3 mg l(-1) atrazine. Our hypothesis was that the denitrifying barrier would remove nitrate from the flowing water and that the downstream reaction would remove atrazine. Our hypothesis proved correct; the two reactor system removed 99.9% of the atrazine during the final 30 weeks of the study. The denitrifying barrier removed approximately 98% of the nitrate and approximately 30% of the atrazine while the aerobic reactor removed approximately 70% of the initial atrazine. The system continued to work when the amount of nitrate-N in the influent water was increased to 50 mg l(-1). A mercury poisoning study blocked the degradation of atrazine indicating that biological processes were involved. An in situ denitrifying barrier coupled with an air injection system or other oxygenation process might be used to remove both nitrate and atrazine from contaminated groundwater or to protect groundwater from an atrazine spill.

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Drinking-Water Nitrate, Methemoglobinemia, and Global Burden of Disease: A Discussion

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The author declares she has no competing financial interests.

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See letter "Infant Methemoglobinemia: Causative Factors" in volume 113 on page A805b.

This article has been cited by other articles in PMC.

Abstract

Go to:

On behalf of the World Health Organization (WHO), I have undertaken a series of literature-based investigations examining the global burden of disease related to a number of environmental risk factors associated with drinking water. In this article I outline the investigation of drinking-water nitrate concentration and methemoglobinemia. The exposure assessment was based on levels of nitrate in drinking water greater than the WHO guideline value of 50 mg/L. No exposure–response relationship, however, could be identified that related drinking-water nitrate level to methemoglobinemia. Indeed, although it has previously been accepted that consumption of drinking water high in nitrates causes methemoglobinemia in infants, it appears now that nitrate may be one of a number of co-factors that play a sometimes complex role in causing the disease. I conclude that, given the apparently low incidence of possible water-related methemoglobinemia, the complex nature of the role of nitrates, and that of individual behavior, it is currently inappropriate to attempt to link illness rates with drinking-water nitrate levels.

Keywords: burden of disease, drinking water, methemoglobinemia, nitrates

The Global Burden of Disease project, coordinated by the World Health Organization (WHO), is an attempt to quantify and compare the level of illness at both world and regional levels. This can be done on a disease-by-disease basis (Murray and Lopez 1996) or in relation to various risk factors such as malnutrition; exposure to poor water, sanitation, and hygiene; or indoor air pollution (Murray and Lopez 1996; Prüss et al. 2002; WHO 2002). Information relating to environmental risk factors, such as the amount of illness attributable to lead in the environment (Fewtrell et al. 2004), can be very powerful in terms of informing policy decisions. Disease

burden, in relation to environmental risk factors, is generally determined by establishing the exposure of the population (on a regional basis) to the chosen risk factor and combining these data with exposure–response relationships for the selected health outcomes to estimate the number of people affected with each outcome. This may then be converted into disability-adjusted life years, accounting for the severity and duration of each health outcome.

Nitrate pollution of drinking water (which has been linked with certain health outcomes) is known to be increasing (Croll and Hayes 1988; Tandia et al. 2000; WHO 1985; Young and Morgan-Jones 1980). WHO therefore considered it useful to determine whether it was possible to establish a disease burden estimate.

Health Outcomes

Go to:

Nitrate is a naturally occurring ion, which makes up part of the nitrogen cycle. The nitrate ion (NO_3^-) is the stable form of combined nitrogen for oxygenated systems. Although it is chemically unreactive, it can be microbially reduced to the reactive nitrite ion. Nitrate has been implicated in methemoglobinemia and also a number of currently inconclusive health outcomes. These include proposed effects such as cancer (via the bacterial production of *N*-nitroso compounds), hypertension, increased infant mortality, central nervous system birth defects, diabetes, spontaneous abortions, respiratory tract infections, and changes to the immune system [Centers for Disease Control and Prevention (CDC) 1996; Dorsch et al. 1984; Gupta et al. 2000; Hill 1999; Kostraba et al. 1992; Kozliuk et al. 1989; Malberg et al. 1978; Morton 1971; Super et al. 1981]. Although the role of *N*-nitroso compounds and nitrite in the promotion of cancer would appear to be incontrovertible, the evidence relating to the role of nitrates is less clear (Pobel et al. 1995). Thus, methemoglobinemia was the only health outcome I considered further in this investigation.

Methemoglobin (MetHb) is formed when nitrite (for our purposes, formed from the endogenous bacterial conversion of nitrate from drinking water) oxidizes the ferrous iron in hemoglobin (Hb) to the ferric form (Fan et al. 1987). MetHb cannot bind oxygen, and the condition of methemoglobinemia is characterized by cyanosis, stupor, and cerebral anoxia (Fan et al. 1987). Under normal conditions, < 2% of the total Hb circulates as MetHb (Fan et al. 1987). Signs of methemoglobinemia appear at 10% MetHb or more, as shown in Table 1 [Craun et al. 1981; Kross et al. 1992; National Academy of Sciences (NAS) 1981]. Symptoms include an unusual bluish gray or brownish gray skin color, irritability, and excessive crying in children with moderate MetHb levels and drowsiness and lethargy at higher levels (Brunning-Fann and Kaneene 1993). Diagnosis is through the observation of chocolate-colored blood or a laboratory test showing the presence of elevated MetHb levels (Brunning-Fann and Kaneene 1993).

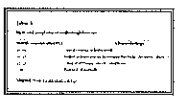

Table 1

Signs and symptoms of methemoglobinemia.

Infant methemoglobinemia was first linked to nitrates in drinking water by Hunter Comly in the United States in 1945. He reported on two cases and concluded that methemoglobinemia may occur in an infant after ingestion of water high in nitrates, especially if the infant was suffering from a gastrointestinal disturbance (Comly 1945). Fan et al. (1987) have noted since then that microbially poor water (i.e., high in microbes) and high drinking-water nitrate levels often go "hand in hand," and gastrointestinal illness, as a result of exposure to poor water quality, may play a role in methemoglobinemia.

Nitrate-related drinking-water methemoglobinemia is principally a disease of young children, with bottle-fed or weaned infants < 4 months of age being the most susceptible. This age group is the most susceptible because of a combination of factors (Ayebo et al. 1997), including:

- A higher gastric pH, which allows greater bacterial invasion of the stomach and hence an enhanced conversion of ingested nitrate to nitrite
- A greater fluid intake relative to body weight

- A higher proportion of fetal Hb (which may be more rapidly oxidized to MetHb than adult Hb)
- Lower NADH-dependent MetHb reductase activity (the enzyme that converts MetHb to Hb).

However, although the gastric pH in infants may be higher than that seen in adults, L'hirondel and L'hirondel (2002) have suggested that the general stomach conditions are still not really suitable for the microbial conversion of nitrate to nitrite.

Exposure Assessment

Go to:

Methemoglobinemia has several causes, as shown in Table 2, including exposure to nitrite or nitrate through the diet (although high dietary nitrate levels are generally accompanied by high nitrite levels). The principal area of interest in this study, however, was drinking water; therefore, the exposure assessment was based on the concentrations of nitrate in drinking water.

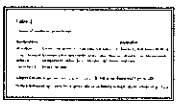

Table 2

Causes of methemoglobinemia.

Guidelines and regulatory limits relating to the amount of nitrate in drinking water, of 10 mg/L nitrate–nitrogen (NO_3–N) and 50 mg/L nitrate [nitrate concentrations are typically expressed either as mg/L NO_3–N or nitrate (NO_3); 50 mg/L NO_3 is equivalent to 11.3 NO_3–N], were established to prevent infantile methemoglobinemia [U.S. Environmental Protection Agency (EPA) 1977; WHO 1958, 1996], and were based principally on the results of a survey conducted by the American Public Health Association (APHA) and reported by Walton (1951). This survey reported on > 270 cases of methemoglobinemia in infants in the United States (for whom nitrate drinking-water levels were available for 214 cases), although APHA emphasized restricting the data to those cases thought to be definitely associated with nitrate-contaminated water. As noted by Walton (1951), no cases were observed with drinking-water concentrations < 10 mg/L NO_3–N. High nitrate for the purposes of the exposure assessment has been taken, therefore, to mean anything exceeding the current recommendations.

Natural levels of nitrate in groundwater depend on soil type and geology. In the United States, naturally occurring levels of nitrate are in the range of 4–9 mg/L. Agricultural activities, however, can result in elevated levels (in the region of 100 mg/L; WHO 1996).

High-nitrate drinking water is most often associated with privately owned wells, especially with shallow wells with depths < 15 m in regions with permeable soils (Fan et al. 1987). It is exactly this situation of small community water supplies, in which poorly regulated and unsanitary waters are found, that could induce gastrointestinal symptoms in consumers (Fewtrell et al. 1998). Shearer et al. (1972) note that the factors responsible for elevated nitrate contents in well-water sources include geography, geology, groundwater hydrology, and the addition of nitrates naturally and from surface contamination by nitrogenous fertilizers or by organic waste of human or animal origin. Although water derived from privately owned wells may be the most common source of high-nitrate drinking water, municipal drinking water supplies may also be contaminated. Vitoria Minana et al. (1991) report on nitrate levels in the Valencia region of Spain, where concentrations exceeded the WHO guideline level (50 mg/L) in 95 towns, with 18 municipalities reporting levels > 150 mg/L.

It has been estimated that 15 million families in the United States receive their drinking water from private wells [U.S. General Accounting Office (GAO) 1997]. Assuming an exceedence rate of 13% (based on a survey of 5,500 wells in nine Midwestern states; CDC 1998), an estimated 2 million household supplies would exceed the federal standard of 10 mg/L NO_3–N. Using current birth rates Knobeloch et al. (2000) estimate that 40,000 infants < 6 months of age are expected to be living in homes with high-nitrate drinking water.

Except for the United States, most literature on nitrate contamination covers small areas and does not allow estimates of the number of people exposed to be calculated.

Exposure–Response Relationship

Go to:

Complex co-factor relationships do not currently allow the establishment of a quantitative exposure–response relationship for human exposure to nitrates in food or water and the subsequent development of methemoglobinemia. Two factors make estimates of the number of cases of methemoglobinemia hard to establish: Generally, methemoglobinemia is not a notifiable disease; and definitions of methemoglobinemia (in terms of the required level of MetHb) vary in the literature. Some authors, however, do report incidence rates.

In three counties in Transylvania (Romania), mean incidence rates varied between 117 and 363 of 100,000 live births (for the full 5 years between 1990 and 1994). These rates, reported by Ayebo et al. (1997), were considerably below the previously reported levels of 13,000/100,000 live births, or 13%, which may reflect a decrease in the availability of cheap inorganic fertilizer (hence a decrease in nitrate contamination levels). In 1985, WHO reported that > 1,300 cases of methemoglobinemia (with 21 fatalities) occurred in Hungary over a 5-year period. Indeed, up until the late 1980s methemoglobinemia was a serious problem in Hungary (Hill 1999). Although there are reports of high nitrate concentrations in drinking water (i.e., > 50 mg/L nitrate) from around the world (Hoering and Chapman 2004), these are rarely paralleled by reports of methemoglobinemia. Where illness has been reported, many of the cases predate the early 1990s, and Hanukoglu and Danon (1996) have proposed that the apparent decline in the incidence of methemoglobinemia is suggestive of an infectious etiology.

Discussion

Go to:

In addition to the problem of limited data (relating to both the population exposed to nitrate in drinking water and the rate of illness), examination of the literature also revealed a number of factors that would either lead to uncertainty in the disease burden estimate (e.g., avoidance behavior) or cast doubt on the validity of the whole exercise.

Limited data. Numerous reports from all over the world describe water supplies (often privately or community-owned wells, rather than municipal supplies) with nitrate concentrations greater than the WHO guideline value of 50 mg/L (Hoering and Chapman 2004). These rarely, however, also include figures on the population supplied by these water sources. Because agricultural and organic waste disposal activities (e.g., through inappropriate sanitation measures) can greatly influence water nitrate concentrations, it is not possible to use geologic data as a possible means to estimate affected population. Thus, it is currently difficult to estimate the population that might be exposed to elevated drinking-water nitrate. Even where the number of people known to have supplies with high nitrate levels can be assessed, this is unlikely to be an accurate estimate of those actually exposed to high-nitrate drinking water. In a number of countries, such as the United States and United Kingdom, health advisories are issued to pregnant women and mothers with formula-fed infants known to be living in high-nitrate areas (Fraser and Chilvers 1981; Schubert et al. 1999). Indeed, Schubert et al. (1999) found that avoidance behavior (i.e., the use of water from another source, such as bottled water or installation of a nitrate removal system) was common, especially in high-risk groups. On the other hand, owners of private wells often boil the water before using it in infant food, an action that, when done excessively, may concentrate nitrate (Ayebo et al. 1997).

A literature review (conducted by searching publication databases and bibliographic lists from collected references) revealed few reported cases of methemoglobinemia linked to water consumption in the last 12 years (Hoering and Chapman 2004). It is possible that because methemoglobinemia is generally not a notifiable disease, there may be under-reporting. It is also possible that there is under-diagnosis, although this is less likely with severe cases, where extensive cyanosis is seen.

Role of nitrate.

Since the 1940s, when the first cases of methemoglobinemia related to drinking water were reported, there has been the suggestion that gastrointestinal upset, and hence infection, may play a role in the development of the disease (Comly 1945). Comly (1945) suggested that it was advisable to use well water containing no more than 10 or, at the most, 20 mg/L NO_3–N for infant feeding. This level seemed to be confirmed by the survey conducted by the APHA [cited by Walton (1951)], which suggested that, in instances where drinking-water nitrate had been determined, there were no cases of methemoglobinemia where water concentrations were < 10 mg/L NO_3–N (~ 45 mg/L nitrate). However, this conclusion would have been influenced by the methodology, which placed an emphasis on cases thought to be linked to nitrate-contaminated water. However, the APHA survey noted that most cases of methemoglobinemia studied were related to NO_3–N concentrations > 40 mg/L. Additionally, Walton (1951) noted a number of factors that may play a role in the development of infant methemoglobinemia, yet at some point a simple role for nitrate became accepted.

It is becoming increasingly clear, however, that the early authors were correct to be cautious, and now it appears that there is an association between gastrointestinal illness and symptoms of methemoglobinemia in the absence of exogenous nitrate exposure (Bricker et al. 1983; Dagan et al. 1988; Danish 1983; Gebara and Goetting 1994; Kay et al. 1990; Lebby et al. 1993; Smith et al. 1988; Yano et al. 1982). Yano et al. (1982) suggested that diarrhea produces an oxidant stress that increases MetHb production and that acidosis impairs the MetHb reductase systems. Nitric oxide, produced by several tissues in response to infection and inflammation, has also been proposed as a possible mechanism (Gupta et al. 1998; Levine et al. 1998), because nitrite is a product of nitric oxide metabolism. Avery (1999) suggested that exogenous nitrates (e.g., through the consumption of drinking water), rather than causing methemoglobinemia, increase its severity. L'hirondel and L'hirondel (2002) suggested that in cases where methemoglobinemia has been associated with infant formula made with drinking water containing elevated nitrate or carrot soup preparations, it is possible that bacterial growth within the bottle or stored soup and exogenous conversion of nitrate to nitrite is the source of the problem.

Conclusions

Go to:

This study did not set out to review the role of nitrates in the causation of methemoglobinemia; however, examination of the literature suggests that a number of authors are starting to question the simple association between nitrate and infant methemoglobinemia, in favor of seeing nitrate as a co-factor in one of several causes of the disease. This factor, coupled with the paucity of data in terms of both population exposure and the level of suspected water-related cases of methemoglobinemia, suggests that attempts to estimate a global burden of disease are currently inappropriate.

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ENVIRONMENTAL WORKING GROUP

Pouring It On: Health Effects of Nitrate Exposure

FEBRUARY 1, 1996

For 50 years, physician and public health professionals have known that exposure to high levels of nitrates cau "blue baby syndrome," a condition caused by lack of oxygen in infants. Thousands of cases of this condition h been reported worldwide since its initial diagnosis in 1945, and the current EPA standard has been set in order protect infants from methemoglobinemia from excessive exposure to nitrate. Unfortunately, in tens of thousan households, infants continue to drink water contaminated with nitrate at levels deemed unsafe by EPA. And qu likely, the current EPA standard does not adequately protect the public health. The current EPA standard of 10 ppm1 is based on a 45 year old survey of methemoglobinemia in infants. Since 1950, however, there have bee number of reported cases of methemoglobinemia caused by nitrate at less than 10 ppm in drinking water (Sattelmacher 1964; Simon 1962).

Comparing EPA's nitrate in water standard with other nitrate standards illustrates just how out of step the wate standard really is.

> In 1980, the USDA established a zero-tolerance for added nitrate in food destined for infants and childre and significantly restricted exposure to nitrate in the overall food supply. Unfortunately, this provides no protection for bottle fed infants during the first three to four months of life. This is precisely the time whe infants, the most vulnerable population, are at peak susceptibility to the toxic effects of nitrate.
>
> In Germany and South Africa the drinking water standard for nitrate is 4.4 ppm -- more than twice as stri the U.S. standard of 10 ppm (Kross, et al. 1995). The European Economic Community has established a health guideline of 5.6 ppm, and studies of infants in Europe have found that three to four percent of methemoglobinemia cases in infants occurred at doses lower than 10 ppm (Sattelmacher 1964; Simon 19 Clearly, health authorities in many other countries believe that nitrate poses an unacceptable risk to infan children below the current EPA standard of 10 ppm.
>
> Most European countries have banned N-nitrosamines and N-nitroso compounds, along with their nitrate nitrite precursors from baby bottle nipples due to concern about exposure to these potent carcinogens ear life (Westin 1990). In the U.S., there is no standard, although Mead Johnson, a large producer of infant formula and infant products including baby bottle nipples, reformulated their nipples to remove all N-nitrosamines and precursors.

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Is Nitrate an Ecologically Relevant Endocrine Disruptor in Vertebrates?[1]

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SYNOPSIS. **The last three decades have brought clear recognition that many populations of animals are experiencing severe declines or local and global extinctions. Many examples have become common knowledge to the general public, such as worldwide declines in amphibian populations and extensive loss of coral reefs. The mechanisms underlying these and other changes are poorly understood. However, a growing literature indicates that a wide array of chemical contaminants have the potential to disrupt normal cell-to-cell signaling mechanisms. A global pollutant of most aquatic systems, nitrate has the potential to be an endocrine disrupting contaminant. This paper reviews studies performed on vertebrates demonstrating that nitrate and/or nitrite have the potential to alter endocrine function. Further, a retrospective study of our work on alligators from various lakes in Florida suggests that nitrate could contribute to some of the altered endocrine parameters previously reported in juvenile animals. We propose hypotheses suggesting that nitrate could alter steroidogenesis by 1) conversion to nitrite and nitric oxide in the mitochondria, the site of initial steroid synthesis, 2) altering Cl^- ion concentrations in the cell by substituting for Cl^- in the membrane transport pump or 3) binding to the heme region of various P450 enzymes associated with steroidogenesis and altering enzymatic action. Future studies are needed to examine the endocrine disruptive action of this ubiquitous pollutant. A growing literature indicates that all biologists studying natural systems, whether they choose to or not, must now consider contaminant exposure as a direct influence on their studies. That is, ubiquitous global contamination has the potential to alter the endocrine, nervous and immune systems of all organisms with resulting changes in gene expression and phenotypes.**

INTRODUCTION

A central focus of comparative physiology and endocrinology has been the influence of environmental factors on the development and performance of various systems or whole organisms. Over the last century, it has been clearly established that such factors as temperature, pH, salinity, photoperiod and gas tensions affect the endocrinology of vertebrates (Norris, 1997). As part of these studies, we have also become aware of the influence of human activities on the biology of numerous species. The endocrine-like actions of various chemical contaminants have been a recent focus of much research (see Ankley *et al.*, 1997; Guillette and Crain, 2000; McLachlan, 2001). These studies have investigated endocrine disrupting chemicals released from industrial activities, sewage treatment works, refuse dumps, confined animal feed operations and agriculture fields (see Noaksson *et al.*, 2001; Orlando *et al.*, 2004; Soto *et al.*, 2004). In addition, pharmaceuticals and other chemicals with endocrine-like activity have been identified in food products and drinking water (Kolpin *et al.*, 2002; Miyahara *et al.*, 2003).

Endocrine disrupting chemicals affect an organism's physiology through a number of mechanisms. They may mimic naturally occurring steroids, act as hormone receptor agonists or antagonists or alter the enzymes responsible for hormone synthesis and degradation (for reviews, see Crain *et al.*, 2000; Gray *et al.*, 2001; Rooney and Guillette, 2000). Using these definitions, synthetic chemicals such as pesticides, plasticizers or industrial chemicals and naturally occurring heavy metals and plant or fungal compounds have been defined as endocrine disrupting contaminants (EDCs). However, absent from this discussion have been the possible endocrine altering roles of ions, such as nitrates and nitrites, that occur at high levels as environmental pollutants (Sampat, 2000).

Each year, it is estimated that the human population consumes approximately 25 million tons of protein nitrogen, a figure expected to climb to 40–45 million tons by 2050 (Jenkinson, 2001). Currently, humans fix 160 million tons of nitrogen per year, of which 83 million tons is used as agricultural fertilizer. The extensive use of fertilizers has dramatically increased the combined nitrogen entering freshwater and estuarine habitats. In addition, an increasing number of reports have identified agricultural nonpoint source pollution as the leading source of water quality impacts to freshwater systems, including freshwater aquifers (Cassman *et al.*, 2002; Sampat, 2000). The USA has the third largest land area under irrigation for food production in the world with 43% of its annual groundwater use going to irrigation (Sampat, 2000). The impact of agricultural practices on groundwater quality is of particular concern because a majority of the population on American farms, both human and livestock, receive their drinking water supply from private wells. Many of these wells are shallow and are vulnerable to water pollution, especially from nitrate (Cassman *et al.*, 2002; Mitchell and Harding, 1996; Sampat, 2000). Waste from animal production and fossil fuels also contribute significantly to environmental nitrogen

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loading. In the United States, nitrate contamination is the major reason public water supplies have been closed. The current public health standard for safe drinking water in the USA requires that nitrate not exceed concentrations of 10 mg/L (ppm) as nitrate-N or 45 mg/L (ppm) as nitrate (10 ppm nitrate-N ≈ 45 ppm nitrate)[3]. When nitrate in a water supply reaches or exceeds these drinking water standards, very costly measures must be taken.

Extensive, and continuing research has been performed on the ecological impacts of nitrogen loading. A large literature exists examining the influences of nitrate and nitrite loading on physiological performance of the vascular and digestive systems of terrestrial vertebrates exposed through food or drinking water, but relatively few studies examine the physiology of vertebrates living in eutrophic aquatic systems. In large part, this lack of study has been due to the belief that inorganic nitrate and nitrite ions affect plant and bacterial life but have little direct physiological impact on multicellular animals, especially vertebrates. A growing number of studies suggest that nitrate and nitrite ions act on many systems and could serve as direct precursors for the production of nitric oxide, a potent physiological regulator in vertebrates. This commentary proposes a hypothesis relating direct disruption of the reproductive endocrinology of wildlife with exposure to nitrate rich, eutrophic freshwater environments. Thus, nitrates could pose a direct threat to the conservation and restoration of vertebrate populations and the ecosystems they depend on for survival.

REPRODUCTIVE-ENDOCRINE ALTERATIONS WITH NITRATES

Nitrogenous compounds have become a major global pollutant in freshwater and estuarine ecosystems. Two compounds with known physiological influence are nitrate and nitrite (Jensen, 2003; Levallois and Phaneuf, 1994; Zraly *et al.*, 1997). Nitrate and nitrite have been reported to be toxic in humans and animals for decades (Avery, 1999). As early as 1945, methemoglobinemia (Blue Baby syndrome) was associated with drinking nitrate-contaminated well water on farms from the Midwest USA. Methemoglobin is formed during nitrate-induced oxidation of hemoglobin. This prevents normal oxygen binding and leads to hypoxia. Methemoglobinemia, as well as additional concerns, continue today with increasing nitrate contamination of ground water (Avery, 1999; Porter *et al.*, 1999). Nitrogen-laden rainwater discharges have also been associated with algal blooms, fish kills and anoxic conditions in many freshwater systems. Although public drinking water from modern utilities falls below the 10 mg/L (ppm) NO_3-N limit, as required by law, contamination of rural water supplies, aquifers, rivers, ponds and farm wells continues to occur (Porter *et al.*, 1999; Sampat, 2000). For example, in one study, 18% of Iowa's private drinking water wells were contaminated with more than 10 mg/L NO_3-N (Kross *et al.*, 1993). In Florida, a number of springs discharging directly from the aquifer have levels >20 mg/L of detectable NO_3-N (Katz *et al.*, 1999).

Mortality and developmental changes with nitrate

The effects of nitrate on various species range from gross toxicity to more subtle changes in physiology and development. For example, mortality of larval cutthroat trout, Chinook salmon, and rainbow trout occurred at nitrate concentrations ranging from 2.3–7.6 mg/L NO_3-N (Kincheloe *et al.*, 1979). Survival of Chorus frog and Leopard frog tadpoles was significantly decreased after exposure to 10 mg/L NO_3-N (Hecnar, 1995). In contrast, the 96-hour LC_{50} (median lethal concentration) for fathead minnow larvae was 1,341 mg/L NO_3-N, whereas it is 462 mg/L NO_3-N for adult *Daphnia magna* (Scott and Crunkilton, 2000).

Metabolic effects of nitrate have been observed in several frog species. Toad tadpoles (*Bufo bufo*) exposed to 11 or 23 mg/L NO_3-N began and completed metamorphosis earlier and grew faster than controls. Exposed toads also tended to have unusual swimming patterns and deformities, including missing or deformed forelimbs and toes (Xu and Oldham, 1997). Cascades frogs (*Rana cascadae*) exposed to 3.5 mg/L NO_2-N metamorphosed more slowly than controls, but emerged from the water at the same time as controls, even though they were less developed (Marco and Blaustein, 1999). These examples suggest that sensitivity to nitrate varies greatly among species and is stage of development dependent. Further, these data suggest that at least one endocrine axis involving the thyroid could be influenced, as metamorphosis and growth in tadpoles is significantly influenced by thyroid hormones.

Thyroid alterations with nitrates

A number of studies have observed alterations in various endocrine parameters associated with dietary or experimental exposure to nitrates. In the early 1950s, iodine uptake in rats was shown to be depressed

[3] ***A note on the reporting of nitrate concentrations***—Nitrate (NO_3) and nitrite (NO_2) concentrations in water are reported using various approaches. Traditionally, water quality for aquacultural settings, surface water, and agricultural runoff has been reported as NO_3-N, or nitrate-nitrogen. This measure actually represents the amount of nitrate and nitrite in the sample, given that the two molecules usually exist in some sort of equilibrium. Generally 95–97% of the NO_3-N will be nitrate, and the remaining 2–5% will be nitrite, although this depends on other factors such as microbial action and fish population density. Some experimental studies report concentrations of NO_2-N because nitrite was specifically dosed. Nitrate-nitrogen (or nitrite-N) represents the concentration of nitrogen molecules only, based on molecular weight. Therefore, the concentration of nitrate (NO_3), for example, is approximately 4.4 × the value reported as NO_3-N because the NO_3 measurement must include the weight of the oxygen. Aquatic nitrogen concentration may also be reported as total nitrogen (TN). Total nitrogen consists of dissolved and particulate organic and inorganic nitrogen, not including N_2 gas. NO_3-N and TN are reported in parts per million (ppm), mg per liter (mg/L) or millimolar (mM) units. In contrast, physiological studies report NOX concentrations, which represent nitrogen associated with nitrate, nitrite, and nitric oxide. Depending on the literature examined, any of the above reporting conventions can be used.

by nitrate, with subsequent alterations in thyroid gland morphology and function (Wyngaarden *et al.*, 1952, 1953). Altered uptake of iodine has been demonstrated in humans (Ellis *et al.*, 1998), domesticated mammals (Pisarikova *et al.*, 1996; Zraly *et al.*, 1997) and fish (Lahti *et al.*, 1985). Lahti *et al.* (1985) also demonstrated that this occurred in multiple fish species and was not limited to iodine uptake by the thyroid—other tissues were affected as well. This effect occurred at ecologically relevant, although elevated, concentrations of nitrate in water (77 mg/L NO_3-N). Adult bulls receiving oral potassium nitrate (100 g/day), also exhibited altered iodine uptake with depressed thyroid gland activity. Indeed, decreased thyroxine concentrations were observed during the period of administration (Zraly *et al.*, 1997). Further, thyrotropin levels were non-detectable for up to 35 days post administration suggesting an influence on the pituitary axis as well. Nitrate (4 g/animal/day in feed) was also goitrogenic in sheep, and their offspring if exposure occurred during pregnancy (Kursa *et al.*, 2000). The goitrogenic effect of nitrate could be suppressed with dietary supplements of iodine. In combination with various herbicides, nitrate also affected thyroxine concentrations in rodents (Porter *et al.*, 1999).

Androgen alterations with nitrates

The studies described above suggest that nitrates can be disruptive of the thyroid axis. Additional studies using rodents or *in vitro* cell culture (mouse Leydig tumor cells—MLTC-1) indicate possible endocrine disruptive actions for dietary nitrates. Inorganic nitrate has been demonstrated to inhibit gonadotropin-induced androgen synthesis from mammalian testicular Leydig cells *in vitro* and androgen synthesis *in vivo* (Panesar, 1999; Panesar and Chan, 2000). These studies followed an observation that nitrate/nitroglycerin therapy in humans influenced circulating androgen concentrations, as well as blood pressure. The *in vitro* study reported a dramatic reduction in Leydig cell steroidogenesis at pharmacological doses of nitrate and nitrite and decreased responsiveness of Leydig cells to gonadotropin stimulation (Panesar, 1999). Drinking water experiments with nitrate (50 mg/L $NaNO_3$) reported that exposed male rodents had significantly decreased circulating T concentrations (Panesar and Chan, 2000). These researchers proposed several mechanisms, including one involving nitrate to nitric oxide conversion (see discussion below).

Oral administration of nitrates to bulls (100–250 g/day/animal) also depressed the function of the Leydig cell during and particularly after the administration period (Zraly *et al.*, 1997). In particular, the Leydig cells of the treated bulls appeared less responsive to gonadotropin stimulation, as reported above for rats *in vitro*. Treated bulls also had increased acid phosphatase activity and reduced fructose concentration in their semen. Further, these bulls showed reduced sperm motility, increased secondary sperm abnormalities, and degenerative lesions in the spermatocyte and spermatid germ layers of the testis (Zraly *et al.*, 1997). These effects are similar to those reported in various human populations worldwide (Toppari *et al.*, 1996).

Nitrates and endocrine disruption in wildlife populations

What is the relevance of the above medical, laboratory or farm based studies? Nitrate and nitrite levels have increased in many aquatic systems worldwide due to human activity. The regulation of nitrogen pollution has become one of the priorities for ground and drinking water managers (Knox and Canter, 1996) and should be a major concern for conservation and ecosystem restoration plans as well. Over the last decade, we have become increasingly aware that environmental contaminants act through multiple mechanisms to alter endocrine functioning in vertebrate and invertebrate species (Guillette and Crain, 2000). One of the most common observations is an alteration in steroidogenesis, in particular androgen synthesis and function in male vertebrates (see Rodgers-Gray *et al.*, 2000). For example, fish obtained downstream from sewage treatment plants not only display elevated yolk protein concentrations in their blood—an estrogenic response—but also exhibit reduced plasma androgen concentrations (Folmar *et al.*, 1996). Our laboratory has also reported alterations in the synthesis, plasma concentrations and hepatic metabolism of androgens in alligators living in eutrophic lakes with exposure to pesticides (Guillette *et al.*, 2000; Gunderson *et al.*, 2001). These data and the many other studies from laboratories worldwide require that we examine factors that are "antiandrogenic," irrespective of mechanism or class of compound (Gray *et al.*, 2001).

Alligators, endocrine disruption and eutrophic lakes

Contaminants can induce or suppress normal endocrine responses. Alligators living in several Florida lakes have endocrine alterations that are worse if the animals remain in a polluted lake *versus* being held in clean water after hatching (Guillette *et al.*, 2000; Rooney, 1998). Examinations of the reproductive and endocrine systems of hatchling and juvenile male alligators from Lake Apopka have demonstrated elevations in plasma estradiol-17β (E_2), reductions in the androgens testosterone and 5α-dihydrotestosterone (T and DHT) and altered thyroxine concentrations. In addition, we have observed morphological abnormalities of the testis, phallus and thyroid (Guillette *et al.*, 1999*b*; Pickford *et al.*, 2000). The alterations in plasma hormone concentrations also occur in females but females display a more complex pattern (Guillette *et al.*, 2000). During the first year of life, female alligators exhibit elevated plasma concentrations of E_2 that then drop to concentrations at or below those reported in reference populations during their juvenile years (Guillette *et al.*, 1999*b*). Altered endocrine parameters in juvenile alligators also occur in other Florida wetlands not associated with significant pesticide spills or point source contamination, such as Lake Okeechobee

TABLE 1. *Regression analyses between mean plasma sex steroid concentrations in juvenile alligators and mean lake water concentration of various nitrogenous pollutants/components in seven Florida lakes.**

Hormone	Sex	Total N	NO_3	NH_4	Organic N
Testosterone	Male	−0.46 (0.09)	−0.34 (0.16)	0.08 (0.59)	−0.24 (0.26)
	Female	**−0.56 (0.03)**	−0.47 (0.09)	−0.19 (0.38)	−0.11 (0.46)
	Both	−0.483 (0.08)	−0.33 (0.18)	0.005 (0.91)	0.06 (0.65)
Estradiol	Male	**0.59 (0.04)**	0.02 (0.74)	0.17 (0.41)	0.31 (0.19)
	Female	0.23 (0.28)	**0.62 (0.03)**	0.005 (0.88)	0.32 (0.18)
	Both	0.21 (0.16)	0.005 (0.98)	0.14 (0.54)	0.08 (0.59)

* The seven Florida lakes examined, include; lakes Woodruff, Apopka, Orange, Monroe, Jesup, Okeechobee, Griffin. Data are presented as r^2 (P value). Information on lakes can be found in Guillette *et al.* (1999*b*). Bold = significant.

and Lake Griffin, FL, USA (Guillette *et al.*, 1999*b*). Importantly, we have previously examined the relationship between plasma organochlorine (OCs) pollutants and steroid hormone concentrations in juvenile alligators and found no relationship (Guillette *et al.*, 1999*a*). Plasma hormone concentrations in juvenile alligators from Orange Lake (low OCs, high total nitrogen) and Lake Apopka (high OCs, high total nitrogen) are similar whereas they differ significantly from concentrations found in alligators from Lake Woodruff (low OCs and total nitrogen). This does not mean that pesticides and other pollutants do not influence the endocrinology of alligators. We have shown that developmental exposure to pesticides alters steroidogenesis in the neonatal gonad at concentrations similar to levels occurring in eggs (Crain *et al.*, 1997). In addition, recent gonadotropin challenge studies have documented significant depression in the testicular steroidogenic response of males obtained from Lake Apopka mothers, but raised in captivity under identical conditions with males originating from a reference lake (Edwards and Guillette, unpublished data). These data demonstrate clear organizational abnormalities. Organization in this context refers to the ordering of tissues and signaling patterns during development (see Guillette *et al.*, 1995). Embryonic organization determines the subsequent capacity of cells and tissues to respond to the variety of stimuli encountered during an organism's life. Response to stimuli is referred to as activation. We hypothesize that the abnormalities seen in the juvenile alligators represent both organizational changes due to embryonic exposure to pesticides coupled with activational modifications due to other pollutants, including nitrate, in the lakes.

A first test—a retrospective analysis

Retrospective studies are seldom used in physiological ecology, but readily used in epidemiological and public health studies because they detect patterns that help direct prospective studies that can identify causal agents. A retrospective analysis of sex steroid concentrations in juvenile alligators from eutrophic Florida lakes suggests that some component of the nitrogenous pollutants could contribute to the problems reported previously.

In the spring of 1995, our group examined the endocrinology of juvenile alligators from seven lakes in Florida (see Guillette *et al.*, 1999*b*). We observed significant differences in plasma androgens and estrogens, as well as altered morphological parameters such as penis size, among the populations of alligators inhabiting the study lakes. At that time, we obtained basic lake water parameters such as water temperature and pH but no data on total nitrogen or nitrate/nitrite concentrations. During the spring of 1995, and other seasons as well, various water quality measurements were documented by other researchers for the seven lakes we studied (data were obtained from the US EPA STORET http://oaspub.epa.gov/storpubl/legacy and St. John's Water Management District). Using the water quality data available for samples obtained in the middle of each lake during the spring of 1995 (the same period in which the alligator samples were obtained), we performed a regression analysis (Table 1) to determine if a relationship existed between mean total nitrogen, nitrate, organic nitrogen, pH or ammonia levels in lake water and mean plasma concentrations of testosterone in juvenile alligators. Water samples collected at the center of lakes are considered a better measurement of nitrogen availability, as values obtained at the marshy periphery of lakes can be dramatically altered due to decaying vegetation and photosynthetic activity.

Results of the regression analysis indicate that mean total nitrogen in lake water was inversely correlated with plasma testosterone (T) concentration in juvenile male (Fig. 1A) and female alligators (Table 1). In males, the relationship does not appear to be linear, but a 2 factor polynomial regression generated a highly significant inverse relationship between plasma T concentration and total nitrogen ($R^2 = 0.81$; $P = 0.037$) and nitrate ($R^2 = 0.9$; $P = 0.01$). For males and females, much of the relationship was driven by the values for Lake Woodruff, the only oligotrophic lake. If Lake Woodruff is removed from the analysis, a negative, linear relationship was seen between plasma T concentrations and total nitrogen concentration ($r^2 = 0.51$; $P = 0.05$) for the eutrophic lakes (Fig. 1B–data shown for males only; results for females are similar). These data suggest that some component of the total nitrogen could influence the regulation of plasma T concentrations. Organic N and ammonium concentrations do not seem to explain the relationship, but nitrate concentrations are suggestive (Table 1).

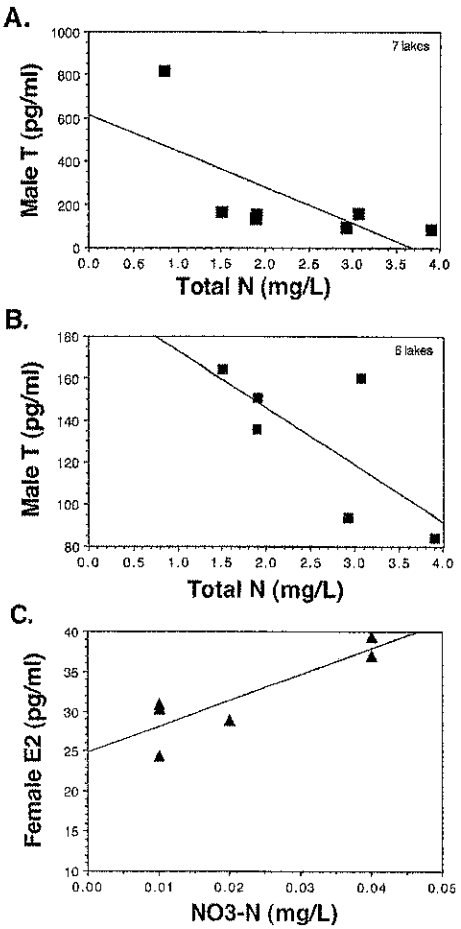

FIG. 1. A. Relationship between lake water total nitrogen and plasma testosterone concentration in juvenile male alligators from seven Florida lakes. B. Relationship between lake water total nitrogen and plasma testosterone concentration in juvenile male alligators from six Florida lakes. The lake removed for panel B is Lake Woodruff, the most oligotrophic lake. C. Relationship between lake water NO_3-N and plasma estradiol concentration in juvenile female alligators from six eutrophic Florida lakes (Apopka, Orange, Monroe, Jesup, Okeechobee, Griffin). Information on the seven lakes (Woodruff, Apopka, Orange, Monroe, Jesup, Okeechobee, Griffin) can be found in Guillette *et al.* (1999*b*).

Converse to results with testosterone, plasma E_2 concentrations exhibited a positive relationship with total nitrogen concentration in males but was weak for females (Table 1). However, a significant positive relationship between mean plasma E_2 and lake water nitrate concentrations was observed among females, even if data from Lake Woodruff were removed (Table 1; Fig. 1C).

As mentioned above, these data suggest the hypothesis that a component of the total nitrogen content of the lake water, or a factor that covaries with total nitrogen content, influences the regulation of androgen and estrogen concentrations in the plasma of juvenile alligators. This analysis was performed to help inform further study and should not be taken as positive support of the hypothesis but must be viewed with caution, as 1) it represents a retrospective study using means and not integrating variation in pollution and hormone levels in each population, and 2) the range over which the nitrate and hormones vary is relatively small. However, it is important to note that these relatively small changes in average hormone concentration in juvenile alligators have been seen repeatedly and were related to significant changes in the morphology of the penis and gonad as well as changes in liver function with regard to enzymes associated with steroid hormone metabolism (Guillette *et al.*, 1999*b*; Gunderson *et al.*, 2001). These data do not provide evidence of a specific mechanism, nor even provide specific associative evidence given that different teams collected the data sets. However, these data indicate that the hypothesis that nitrates can alter steroid regulation warrants further study. Prospective studies examining lake water concentrations matched with plasma and urine nitrate concentrations from specific animals are needed so that variation within and between populations can be included in an analysis. The current analysis among populations indicates that 45% of the variance or more in plasma sex steroids can be explained by either total nitrogen or nitrate concentration in lake water. Within a population, serum OC concentration explains less than 15% of variance in plasma sex steroid concentrations in juveniles (Guillette *et al.*, 1999*a*). It should be noted that similar statistical analyses comparing plasma T or E_2 concentrations in juvenile alligators to other lake water quality factors such as dissolved oxygen, total phosphate, pH or temperature showed no relationships.

NITRATES AND STEROIDOGENESIS: POSSIBLE MECHANISMS

Nitrate and nitrite ions are highly soluble in water and form water-soluble salts with many cations, like potassium and sodium. Dietary intake of nitrate results in ready absorption by the proximal small intestine (Walker, 1996). Transport across the membrane is apparently controlled by a nitrate/H^+ cotransporter (Chow *et al.*, 1997). This cotransporter system could have special implications for some of the endocrine actions reported below. Dietary nitrate and nitrite are used by oral and gastric bacteria to synthesize nitric oxide, implicated in gut physiology and immune functions (Benjamin *et al.*, 1994; Walker, 1996). Nitrate is readily found in the plasma, can be found in sweat, rarely occurs in the feces, and is primarily excreted by the mammalian kidney in the urine as nitrate or urea (Ellis *et al.*, 1998). Plasma and urine concentrations increase with dietary intake of nitrate (see Ellis *et al.*, 1998).

In 1995, it was reported that vertebrate mitochondria are capable of NO synthesis via 'non-enzymatic'–non nitric oxide synthase (NOS) activity (Zweier *et al.*, 1995). Since those initial studies, a growing literature supports the observation that mitochondria obtained from vertebrates under stressful conditions can produce NO using nitrite as a precursor (Cadenas *et al.*,

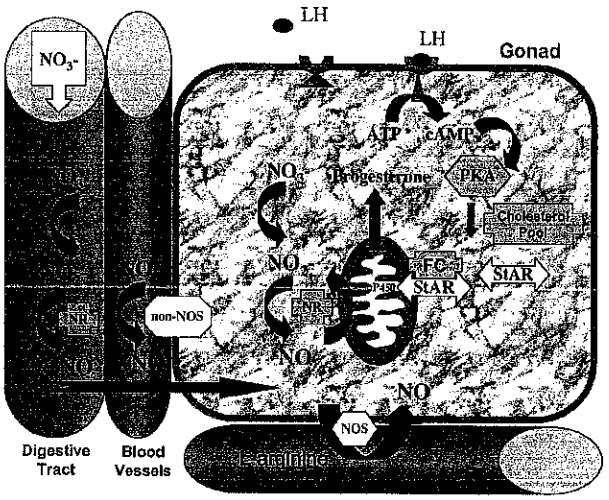

FIG. 2. Schematic hypothesis for the role of nitrate (NO_3-) and nitrite (NO_2-) in nitric oxide (NO) generation in gonadal tissue. NO_3- and NO_2- enter via the gut and are transported to the gonad via the vascular system. Gut bacteria have nitrite reductase (NR) activity and can generate NO from NO_2- locally. It has also been suggested that NR activity is present in mitochondria and endoplasmic reticulum, although several studies indicate that NO_2- can be converted to NO without enzymatic action. NO is readily generated from L-arginine by nitric oxide synthase (NOS). NO has been shown to inhibit gonadotropin (LH) induced steroidogenesis via its actions on the Steroid Acute Regulatory protein (StAR) or the enzyme $P450_{scc}$ (see text). This figure is modified from Panesar and Chan, 2000.

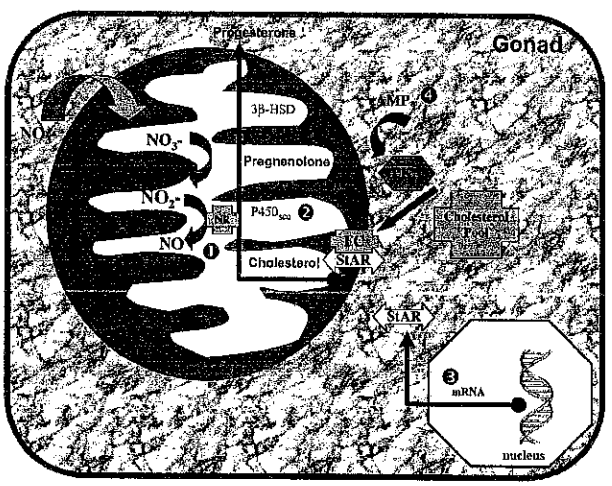

FIG. 3. Schematic representation of actions in the mitochondria of steroidogenic cells in the gonad. Protein kinase A is activated by cAMP, freeing cholesterol from the cytoplasmic pool. Free cholesterol (FC) is transported across the mitochondrial membrane by Steroid Acute Regulatory Protein (StAR). This cholesterol serves as the precursor for steroidogenesis and the enzymes $P450_{scc}$ and 3β-HSD produce pregnenolone and progesterone that is released back into the cytoplasm. Progesterone serves as a precursor for the production of androgens and estrogens. Nitrate (NO_3-) has the potential, either by direct action or via the production of nitric oxide (NO) ❶, to alter P450 enzyme activity ❷, alter gene expression and thus transcription or translation of StAR ❸ or alter cAMP production ❹ by altering cytoplasmic ion concentrations, such as modifications in chloride ion concentration.

2000; Kozlov *et al.*, 1999; Lepore, 2000). Nitric oxide is a potent cellular signal, used in a wide variety of regulatory physiological pathways. The basis for the non-NOS synthesis of NO is still under intense study and debate exists as to whether this phenomenon occurs in healthy tissue. It has been suggested that other enzymes can generate NO from nitrate (see Meyer, 1995; Nohl *et al.*, 2001; Nohl *et al.*, 2000). Zweier and colleagues have shown that a change in pH (7.4 to 5.5) alone is capable of generating NO from nitrite in biological tissues (Samouilov *et al.*, 1998; Zweier *et al.*, 1999). In short, nitrites could be used *in vivo* to generate NO and other reactive oxides that would be disruptive to cellular function (Fig. 2). Multiple mechanisms could be involved depending on the tissue, physiological state of the organism and environment. Well established actions of NO have been observed on steroidogenesis in humans, laboratory species and cell cultures (for examples, see Del Punta *et al.*, 1996; Kostic *et al.*, 1998; Van Voorhis *et al.*, 1994; Weitzberg and Lundberg, 1998). Both stimulatory and repressive roles of NO have been described.

Alternative hypotheses could also explain the observations related above. For example, alterations in chloride ion concentration in steroidogenic cells could alter hormone synthesis as 1) nitrate is known to substitute for chloride in the chloride-bicarbonate membrane transporter system and 2) chloride ion concentrations are known to influence steroidogenesis (depletion augments steroidogenesis [Cooke *et al.*, 1999; Panesar, 1999; Ramnath *et al.*, 1997]). This latter observation could explain the positive relationship between lake water nitrate and estradiol observed in juvenile alligators. However, it does not explain the depression in testosterone.

The molecular and cellular activities driving steroidogenesis have been studied intensely. These include the activity of several factors regulating steroid synthesis, such as the steroidogenic acute regulatory (StAR) protein and the enzyme $P450_{scc}$ (Fig. 3). StAR regulates entry of cholesterol (the basic steroid hormone precursor) into the mitochondria, prior to steroidogenesis (Stocco and Clark, 1996). $P450_{scc}$ modifies cholesterol in the mitochondria prior to the formation of pregnenolone, which is converted to progesterone and subsequently all other steroid hormones (Fig. 3). Several reports indicate that pesticides can alter transcript levels of StAR and $P450_{scc}$ as well as modify the cellular activity of these proteins (Stocco and Clark, 1996; Walsh and Stocco, 2000; Walsh *et al.*, 2000). NO has also been shown to alter StAR expression and activity. Furthermore, we have observed that steroidogenesis was depressed in the MA-10 human Leydig cell line following exposure to nitrate and nitrite exposure (Stocco and Guillette, unpublished data). In addition, nitrate, nitrite and NO could influence the action of cytochrome $P450_{scc}$ and other P450 enzymes, (essential for steroidogenesis), by binding to the heme group that characterizes all enzymes of the

P450 superfamily (Danielson, 2002). When NO binds to the heme group it usually inhibits enzymatic action (White *et al.*, 1987). These data suggest that alterations of enzymatic activities of various P450s in the liver and gonad could also be the basis for our observations. That is, it is plausible to hypothesize that observations of altered plasma steroids in contaminant-exposed juvenile and adult alligators are, in part, a response to nitrogenous pollutants. In summary, we suggest that nitrate/nitrite exposure can act directly on steroidogenesis, especially those steps occurring in the mitochondria as well as steroid biotransformation activities in the liver. The relationships presented here are illustrated in Figures 2 and 3.

Future studies need to examine the interaction between nitrate pollution of aquatic systems and the physiology of the organisms living in those systems. It is very likely that future studies will document direct effects of these pollutants on the endocrine systems of organisms. Given the central role of the endocrine system in reproduction, behavior, immune function, growth and metabolism, it is essential that we begin broad scale studies. Nitrogen pollution will continue to grow during this century as human populations grow.

ECOTOXICOLOGY, CONSERVATION AND THE FUTURE

Over the last decade, it has become increasingly obvious that the outcomes and conclusions from studies of wild populations, whether vertebrates or invertebrates, can be influenced dramatically by the contaminant exposure history of the animals under study. These studies, some of which have been presented above, document the sublethal effects of contaminants on the cellular signaling mechanisms of animals (McLachlan, 2001). They suggest that researchers examining the biology of any and all species should investigate the contaminant history of the ecosystem they work in. In short, all biologists studying natural systems, whether they choose to or not, must now consider contaminant exposure as a direct influence on their studies. That is, ubiquitous global contamination has the potential to alter the endocrine, nervous and immune systems of all organisms with resulting changes in gene expression and phenotypes. Contaminants act as one major factor by which the environment alters the expression of the genotype and thus the phenotype (Fox, 1995; Iguchi *et al.*, 2001). These changes are associated with altered reproductive potential, developmental pathways, behavior and survival. Conservation biology, with its goal of preserving and restoring populations and ecosystems, must actively embrace this new world view, and incorporate an understanding of the multiple mechanisms by which contaminants alter the biology of organisms, populations and ecosystems. To do less, would be to exclude a major factor influencing the potential success of such programs.

As we move forward with our studies, we must continue to appreciate that laboratory or even microcosm studies do not represent the temporal and spatial complexity in contaminant exposure seen by the organisms we study. It has become increasing obvious that future studies must examine the organizational as well as activational roles of pollutants (see Guillette *et al.*, 1995). The use of the organization/activation concept, originally derived from the study of neuroendocrinology and behavior, to understand the possible influences of chemical contaminants on the biology of organisms, is just one example of the future need for truly integrative biology. Toxicology is a science of integration, using concepts and theory from the fields of chemistry, biochemistry, physiology, genetics, population biology and probability mathematics, to name but a few. Modern ecotoxicology must embrace our growing understanding of the complexity and variation associated with the biology of organisms, from genomic to ecosystem levels of organization. To do less, would guarantee that we continue to underestimate the impact of chemical pollution on the world's ecosystems.

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CASE REPORT

Extreme methaemoglobinaemia secondary to recreational use of amyl nitrite

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INTRODUCTION

Haemoglobin is continuously oxidized from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) form and reduced back again. The ferric (Fe^{3+}) form, termed methaemoglobin (MetHb), is incapable of transporting oxygen. In the normal physiological state the concentration of methaemoglobin is less than 1%. Figure 1 illustrates the physiological reactions responsible for the reduction of MetHb back to Hb. It is reported that MetHb levels of 10–25% produce cyanosis, 35–40% produce mild symptoms (e.g. dyspnoea), levels of 60% produce lethargy and coma and levels of 70% or more are lethal.[2,3] A case of extreme, life-threatening methaemoglobinaemia due to the recreational use of amyl (isobutyl) nitrite is presented. No case has been found in the literature where the MetHb level was so high.

Key words: amyl nitrate, methaemoglobinaemia, methylene blue

CASE REPORT

A 44-year-old man was brought by ambulance to St Vincent's Hospital Emergency Department at 23.37 hours. The patient had been found in the steam room of a bathhouse, unconscious, blue and lying in a pool of vomitus. There was an empty bottle of amyl (isobutyl) nitrite next to him, and workers at the establishment stated that he had 'consumed' large amounts of amyl nitrite.

On arrival of the paramedics, the patient was hypoventilating, hypotensive, unresponsive to pain and had poor skin colour. There was no response to 2 mg of naloxone, administered intravenously. The patient was intubated endotracheally by the paramedics, given 100% oxygen, ventilated manually and transported to hospital.

On arrival at the Emergency Department his skin was noted to be a deep charcoal grey colour, despite the fact that he was receiving 100% oxygen and having good air entry into both lungfields on

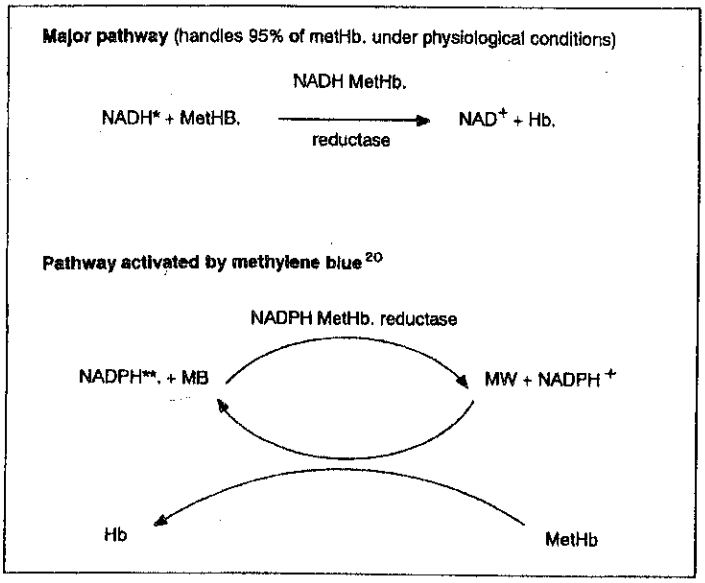

Fig. 1. Mechanisms for reduction of methaemoglobin. MB, methylene blue; MW, methylene white; *Embden-Meyerhoff pathway is the major source of NADH in red blood cells; **hexose mono-phosphate shunt is the major source of NADPH. G6PD is required for its production.

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auscultation. His systolic blood pressure was 70 mmHg, and his pulse was 60 beats min^{-1}. He was unresponsive to pain, and his pupils were constricted, equal and fixed.

Arterial and venous samples were drawn for arterial blood gas (ABG) analysis, MetHb, full blood count (FBC), and determination of electrolytes, urea and creatinine levels.

However, 12 min after arrival the patient suffered a bradycardic arrest, requiring cardio-pulmonary resuscitation and 2 mg adrenaline, administered intravenously. He regained a cardiac output after 30 s. The patient's MetHb level was 94% (n< 1.5%) and he was given 80 mg (c.1 mg kg^{-1}) of tetramethylthionine (methylene blue) intravenously over a period of 10 min. Twenty minutes after administration of the methylene blue, the patient's colour had improved slightly and there was a return of spontaneous respiration, but he remained unresponsive to pain.

Further physical examination revealed a core temperature of 32.5° C (per rectum). There were no focal neurological signs, and the systolic blood pressure remained low (70 mmHg). The abdomen was soft and not distended. There was no sign of trauma, and no other abnormality was found.

The results of initial investigations were as follows (ranges in parenthesis): ABG (FiO_2=1) pH 7.17, (7.35-7.45); Po_2, unable to be measured due to technical problems; Pco_2, 39 mmHg (32–45); HCO_3, 14 mmol L^{-1} (24–31); and base excess (B.E.) –14 (–3-+3). Chest radiograph was normal. A 12-lead electrocardiograph showed sinus rhythm, rate 98 beats min^{-1} with ST elevation (concave down) in leads V1–V6. Hb was 13.1 g dL^{-1} (13.0–18.0), WCC was 9.7×10^9 L^{-1} (4.0–11.0), and platelet count was 211 $\times$ 10^9 (150-400). Blood alcohol level was 0.15%.

A repeat MetHb measurement gave a value of 26%. The patient was given another 100 mg of methylene blue at this time. A further 50 mL of 8.4% sodium bicarbonate were administered for a persisting metabolic acidosis (pH, 7.05; Pco_2, 46; Po_2, could not be measured; HCO_3, 13; B.E., -19).

By 06.00 hours, the patient's colour was pink. A repeat MetHb level was 1.6% at 09.30 hours. At this time the patient was opening his eyes to speech and obeying commands. He continued to improve and was extubated at 18.00 hours. He was confused initially, but his sensorium had cleared by the next morning. He remembered no details from the night of his overdose. Thirty-six hours after admission he was transferred from the intensive therapy unit to the ward.

It was decided to keep the patient in hospital and monitor him for haemolytic anaemia. However, the patient discharged himself, against medical advice, on day 5. There was no evidence of anaemia at this time, and he showed no evidence of any residual neurological deficit on clinical testing. Total creatinine phosphokinase (CPK) levels 12 and 36 h after admission were 385 and 681 U L^{-1}, respectively (<130 U L^{-1}). The MB fractions at these times were 24 and 25U L^{-1}, respectively (<15 U L^{-1}). A repeat ECG before discharge was normal. Clinically, the patient had made a complete, uncomplicated recovery. Further follow-up was performed by the patient's local medical officer. The patient refused counselling from the drug and alcohol service.

We note that the patient presented again to our Emergency Department 6 months later with chest pain after injecting cocaine intravenously. ECG on this occasion was normal, and he was discharged for follow-up with his local medical officer.

DISCUSSION

Although rare, it is important to diagnose methaemoglobinaemia when it presents because it is potentially fatal and yet readily treated. A clue to the diagnosis is the appearance of chocolate brown blood upon venesection or arterial sampling.[1,4,14] The diagnosis can be confirmed by spectrophotometric analysis of the patient's blood, giving a MetHb level expressed as a percentage of the total Hb level. The blood should be analysed very soon after being drawn, as the MetHb level in the sample will decrease with time.[36]

Aetiology

Table 1 lists the agents that most commonly cause acquired methaemoglobinaemia. It has been reported that ingestion of nitrites is not dangerous because they are degraded in the GIT.[41] However, there are many case reports of severe, sometimes fatal, methaemoglobinaemia resulting from ingestion of isobutyl nitrite.[3,16,19,20]

Management of acquired methaemoglobinaemia

After ensuring and protecting the airway, providing appropriate respiratory and cardiovascular support and decontamination (e.g., removing soiled clothing or decontaminating oxidants in the gut), tetramethylthionine (methylene blue, MB) should

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Long-Term Effects of Environmental Endocrine Disruptors on Reproductive Physiology and Behavior

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Abstract

Go to:

It is well established that, over the course of development, hormones shape the vertebrate brain such that sex specific physiology and behaviors emerge. Much of this occurs in discrete developmental windows that span gestation through the prenatal period, although it is now becoming clear that at least some of this process continues through puberty. Perturbation of this developmental progression can permanently alter the capacity for reproductive success. Wildlife studies have revealed that exposure to endocrine disrupting compounds (EDCs), either naturally occurring or man made, can profoundly alter reproductive physiology and ultimately impact entire populations. Laboratory studies in rodents and other species have elucidated some of the mechanisms by which this occurs and strongly indicate that humans are also vulnerable to disruption. Use of hormonally active compounds in human medicine has also unfortunately revealed that the developing fetus can be exposed to and affected by endocrine disruptors, and that it might take decades for adverse effects to manifest. Research within the field of environmental endocrine disruption has also contributed to the general understanding of how early life experiences can alter reproductive physiology and behavior through non-genomic, epigenetic mechanisms such as DNA methylation and histone acetylation. These types of effects have the potential to impact future generations if the germ line is affected. This review provides an overview of how exposure to EDCs, particularly those that interfere with estrogen action, impacts reproductive physiology and behaviors in vertebrates.

Keywords: bisphenol, genistein, soy, estrogen receptors, development, sexual differentiation

Introduction

Go to:

Growing awareness of the prevalence of environmental compounds, both synthetic and naturally occurring, with endocrine disrupting properties has generated considerable debate among scientists, regulatory agencies, and the general public about the potential long-term risks they pose for human and wildlife reproductive health. Is the endocrine disruption hypothesis plausible? Epidemiological evidence that human reproductive health is declining, particularly in Western nations, continues to mount. For example, sperm counts in

Western countries appear to have declined by half in the past 50 years (Carlsen et al., 1992; Swan et al., 2000). In Demark, it is now estimated that more than 10% of men have sperm counts in the infertile range and up to 30% are in the subfertile range (Joensen et al., 2008). There are also indications that female fecundity is declining, even among young women, although the rate and degree to which this is occurring has been difficult to quantify (Brannian and Hansen, 2006; Frey and Patel, 2004; Nyboe Andersen and Erb, 2006). Within the United States, median age at menarche, first breast development, and sexual precocity has steadily advanced, especially among minority populations (Herman-Giddens et al., 1997; Partsch and Sippell, 2001). Similar trends have been noted in Europe and among children adopted from developing countries by Western parents (Aksglaede et al., 2009; Parent et al., 2003; Proos et al., 1991). The cause is likely complex and multi-faceted, but rapidity of the increase in reproductive and behavioral disorders suggests an environmental component. Whether or not endocrine disrupting compounds (EDCs) could be a contributing factor remains the subject of intense scrutiny and other determinants such as diet, stress, and body weight likely also play a role. At issue are both the degree to which low dose exposures to compounds with low hormonal potency can produce appreciable effects in the vertebrate reproductive system, and the difficulty of adequately assessing the potential long term risks of compounds with sex-, life stage-, and tissue-specific effects. Both issues are difficult to address experimentally because the timing, duration and level of human exposure are often uncertain. Moreover, the latency between EDC exposure and the emergence of consequential health effects can be considerably long, even decades, and the degree to which gene-environment interactions can produce inter-individual variability is poorly understood. Finally, predicting human responses from sentinel wildlife cases and from *in vitro* and animal tests of endocrine action is not straightforward. Nonetheless, there is reasonable and increasing evidence from *in vitro* and animal studies to suggest cause for concern. This review will address the potential long term physiological and behavioral effects of exposure to environmental endocrine disruptors in vertebrates, the evidence for human risk, and how this issue has transformed both endocrinology and toxicology.

An EDC is defined (in part) by the United States Environmental Protection Agency (EPA) as, "an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects....at the level of the organism, its progeny, and populations or subpopulations of organisms." This definition includes disruption of lactation, sexual maturation, the ability to produce viable, fertile offspring, sex specific behavior, and premature reproductive senescence. To date, the EPA has identified hundreds of compounds that fit this definition and thousands of others are suspected of having similar properties[1] (Crisp et al., 1998; Toppari et al., 1996). Some of these compounds, such as oral contraceptives and a subset of pesticides, were specifically developed to target the endocrine system but the vast majority of chemicals on the EPA's list were neither designed nor intended to, especially in mammals. Some, such as DDT or the pyrethrins were developed to kill mosquitoes and other pests that spread serious and in some cases life threatening human diseases such as malaria. Others were devised as flame retardants, to kill weeds, or to make plastics harder, clearer, and more resistant to heat stress (bisphenol-a) or more pliable (the phthalates). Compounds produced in nature rather than by humans, such as the phytoestrogens, also fit this definition (Figure 1).

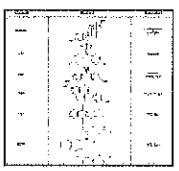

Figure 1

Chemical structures and uses of common endocrine disruptors. DES, bisphenol-a and genistein are classified as estrogen agonists while both of the phthalates are androgen antagonists. DDT is classified as both an estrogen agonist and an androgen antagonist. ...

DDT: The First Known Endocrine Disruptor

Go to:

The discovery that chemicals could interfere with the endocrine system in non-target species was first made by wildlife biologists who noted rapid population declines and abnormal reproductive physiology and behavior in multiple species. For example, as early as the 1930s, famed Florida naturalist Charles Broley noticed abnormal

courtship behavior, reduced nesting behavior, and diminishing birth rates among numerous bird species all across the United States and Canada, most notably bald eagles. His behavioral observations ultimately led him to hypothesize that heavy consumption of fish tainted with the widely used pesticide dichlorodiphenyltrichloroethane (DDT) was sterilizing the birds (Beans, 1996). This contention launched an unprecedented investigation by scientists who both doubted and embraced his hypothesis. It was ultimately determined that DDT and its metabolites, although they did not cause sterility (sperm counts in the birds were normal), feminized male embryos, weakened eggshells, and interfered with reproductive behavior to such a significant degree that it was decimating bird populations. These and similar cases were eloquently documented in the instant best selling book *Silent Spring* by Rachel Carsen (1962), the publication of which launched the modern environmental movement. She postulated that by liberally spraying pesticides in our zealous determination to destroy "pests," we were risking the systemic destruction of ourselves and our environment. This thesis fundamentally changed the public's perception of pesticide use and a decade later, DDT was ultimately banned in the United States largely as a result of public pressure.

The dramatic DDT story demonstrated that exposure to a compound not designed to interact with the endocrine system could induce profound reproductive deficits in non-target species. Despite this however, the capacity for DDT and its metabolites to impact human health is still widely disputed, particularly when exposure occurs at "low levels" rather than through an industrial accident or other large scale mechanisms. At high doses DDT is a potent neurotoxin, but numerous studies in laboratory animals, and *in vitro* assays have established that at low doses it can act as an endocrine disruptor. Even though DDT is no longer used in the US, human exposure to DDT and its metabolites is ongoing. For example, the primary metabolite of DDT, DDE, is far more persistent than the parent compound and thus still found in the environment at low levels. It is also highly lipophilic and accumulates in fat, so body burden increases with age. In addition, DDT is still used in many parts of the world, especially where the risk of contracting malaria, a disease which kills more people than cancer, heart disease or the HIV virus, is great. According the World Health Organization (WHO) nearly a million people died of malaria in 2006, 91% of them children[2]. In contrast, no one is known to have died from routine DDT use. Whether or not DDT can induce disease or impair reproductive development in humans remains the subject of investigation and a controversial topic. A recent epidemiology study associated *in utero* or neonatal exposure with an increased risk of breast cancer (Cohn et al., 2007) a study which adds to a body of literature suggesting that prepubertal exposure to DDT may increase the risk of breast cancer (Clapp et al., 2008). Unfortunately many of the epidemiological studies exploring a potential association between DDT exposure and breast cancer risk have methodological weakness which make the data difficult to interpret, and many scientists remain skeptical that DDT exposure is a causative factor for breast cancer development (Beard, 2006). Emerging evidence from areas where DDT is still used suggests that DDT exposure might also be associated with other diseases including preterm birth, early pregnancy loss, reduced semen quality, disrupted menstruation, and problems with lactation (Beard, 2006; Rogan and Chen, 2005; Venners et al., 2005). But other factors, such as unreliable access to clean water and adequate nutrition are potential confounds and birth rates in these areas remain high. Nursing infants from these regions are frequently exposed to levels in breast milk which exceed the acceptable daily intake of 20 μg/kg body weight per day established by the WHO. Therefore the potential reproductive risk of DDT exposure is ongoing for many populations outside the US.

DDT, its metabolites, and the majority of other endocrine disruptors are now known to affect the endocrine system by multiple mechanisms, most notably by acting as weak estrogen agonists. Thus, the hypothesis that endocrine disruption is a significant health concern for humans would be strengthened by the demonstration that exposure to synthetic estrogens, particularly early in development, can have lasting effects. Unfortunately, this link has already been made.

Evidence of the Potential for Estrogenic Endocrine Disruptors to Affect Human Health Go to:

The clearest evidence that exposure to estrogen during fetal development can impact human reproductive health emerged from the widespread use of the potent synthetic estrogen diethylstilbestrol (DES, Figure 1). Beginning in 1938, DES was initially prescribed to prevent miscarriage but ultimately advertised and dispensed to pregnant women in general to produce "stronger babies" and even administered to newborns to enhance weight gain (Karnaky, 1953; Kuchera, 1971; Palmlund, 1996; Smith, 1948). It is estimated that DES was taken by four to six million (and possibly as many as 10 million) pregnant women in the US alone before human use was suspended in 1971 (Giusti et al., 1995). Non-medical use of DES was also common and thus a significant source of DES exposure, albeit in lower does. For example, it was frequently used in cosmetics, lotions, shampoo, and as a growth promoter in chicken and cattle. DES implants in poultry were outlawed in 1959 when DES residues were found in chicken liver, however this did not affect the use of DES in cattle, which remained ubiquitous until it was ultimately phased out in 1979. By the 1980s, more than 80% of US cattle were estimated to have been exposed, and considerable release of DES into the environment through feed lots and cattle waste has been well documented (Metzler, 1981; Zervos and Rodricks, 1982).

Unfortunately DES was ineffective at preventing miscarriage (Dodds et al., 1938) but the reproductive consequences to the developing offspring (both male and female) would ultimately prove to be extensive. One consequence of *in utero* DES exposure was first identified in 1971 by a group of keen eyed physicians who noticed that girls born to mothers who took DES during pregnancy (collectively referred to as "DES daughters") were more likely to develop an extremely rare type of cervicovaginal clear-cell adenocarcinoma (CCAC) (Herbst et al., 1970, 1971). One in 1000 DES daughters is now estimated to have developed CACC by the age of 34 (Giusti et al., 1995; Rubin, 2007) which is an extraordinarily high rate for such a rare form of cancer. DES exposure is also associated with increased incidences of vaginal dysplasia, vaginal and cervical adenosis and abnormalities of the cervix, vagina, and uterus. DES daughters suffer from reduced fertility, a higher risk of infertility, and more complicated and unsuccessful pregnancies. Complications include ectopic pregnancy, late spontaneous abortions and premature delivery (Palmlund, 1996; Palmlund et al., 1993). Increased rates of psychiatric disorders including depression, anorexia, phobias and learning disabilities have also been reported (Vessey et al., 1983). Damage from DES exposure is not limited to females. DES sons are also affected and show elevated rates of urogenital malformations, undescended testes, and testicular cancer. Low sperm density and mobility has also been observed (Gill et al., 1976; Palmer et al., 2005; Stenchever et al., 1981; Wilcox et al., 1995).

Most of the reproductive outcomes following fetal exposure to DES were predicted by or replicated in animal models (McLachlan et al., 1982; Newbold, 2008; Newbold and McLachlan, 1982). Thus, this unfortunate event in human medical history illustrates both the vulnerability of the developing fetus to estrogenic endocrine disruptors and the importance of animal models for predicting potential adverse effects in humans. Similarly, experimental work in multiple species was equally critical for establishing a causal link between DDT exposure and abnormal reproductive behavior accompanied by compromised fertility in wildlife populations (Beans, 1996; Guillette and Gunderson, 2001; Toppari et al., 1996). Therefore the importance of animal models in human risk assessment paradigms should not be underappreciated.

Key Concepts of Endocrine Disruption: Timing, Dose and Nonlinear Effects

Go to:

The impact of the DES tragedy on the field of toxicology was transformative and three key principles of endocrine disruption emerged. First, the latency between fetal insult and the manifestation of physical or behavioral dysfunction can be extremely long, even decades. This concept had long been recognized by behavioral endocrinologists including Beach, Young, Goy and others exploring the mechanisms by which fetal hormone exposure could alter sexual behavior and sex specific neuroendocrine feedback systems (Balthazart et al., 1996; Gorski, 1963; Goy and Resko, 1972; Marler, 2005; Swaab and Hofman, 1984; Young et al., 1964), but was a breakthrough for toxicologists. It is now the basis for a conceptual framework termed the "fetal basis of adult disease," an idea that has transcended the endocrine disruption field. The concept that exposure to environmental factors, including toxicants, during fetal or neonatal life can interact with the genome and

influence diseases which emerge years later including cancer, infertility, precocious puberty and obesity is potentially revolutionary and is currently a "hot topic" of research.

A second key concept of endocrine disruption is that the dose response of many hormones and EDCs appears to be nonmonotonic. This paradigm is anathema to the fundamental toxicological principle that "the dose makes the poison." The standard approach in toxicology, for experiments looking at acute exposure, is to expose either cells or animals to a few, generally high, concentrations of a given chemical. Selection of those doses is traditionally based on one of two standardized measurements called the LD_{50}, which is the dosage lethal to 50% of the exposed organism or cells, and the lowest observed effect concentration (LOEC), which is the lowest concentration that produces readily observable morphological deformities. Similar designs using high (often near-lethal) doses that are not considered environmentally relevant are frequently used for subchronic and chronic exposure studies as well. These approaches assume that adverse effects increase proportionally and predictably with exposure level (generally linear or sigmoidal) and there is a threshold below which no effect is observed (termed the no observable adverse effect level or NOAEL). A level 1000-fold lower is then deemed the "safe" or "reference dose" for humans. With this model, potential low dose effects can be extrapolated from studies where doses in the range of the LD_{50} (or LOEC) are used. It also assumes that the observed effects will be obvious and include readily appreciable abnormalities. Unfortunately, there is growing evidence to suggest that the effects produced by hormones and many endocrine disruptors are not adequately predicted by this model because they have nonmonotonic dose responses that more closely approximate a U-shaped or inverted U-shaped curve (Andersen et al., 1999; Hayes et al., 2002; Sheehan, 2006; Vandenberg et al., 2006). In addition, effects, as seen with DES and CCAC, are likely to be more insidious and thus potentially difficult to readily identify. It is unclear how compounds can produce U-shaped dose effects but this response likely reflects an integration of two different mechanisms of action, each of which occurs at a different dose range (Vandenberg et al., 2009). Thus a compound like DDT could potentially interfere with estrogen (or androgen) action at low doses but act as a potent neurotoxin at levels closer to the LD_{50} (Toppari et al., 1996). It should be noted that although some toxicologists have expressed concern that nonmonotonic dose responses are underappreciated (Calabrese, 2001; Calabrese and Baldwin, 2003), others doubt their existence (Crump, 2001) and the concept remains highly contentious (Lutz et al., 2005; Melnick et al., 2002; Vandenberg et al., 2009).

A final principle of endocrine disruption made evident by the DES story is that the timing of exposure is crucial. The presence and extent of disorders common to DES sons and daughters varies substantially depending on the timing of the mother's first exposure, total dose, and length of exposure (Faber et al., 1990; Robboy et al., 1981, 1984). In humans and animals, there are critical windows of development, both for the reproductive organs and the brain, when sensitivity to hormones and EDCs is heightened. Again, this was not a novel concept to neuroendocrinologists but was previously underappreciated by toxicologists. Within this concept is also the recognition that the placenta is not impenetrable to EDCs and that, on the contrary, most EDCs likely reach the developing fetus. Maternal estrogens are effectively sequestered by α-fetoprotein but most estrogen-like compounds only weakly or fail to bind to α-fetoprotein and can therefore enter fetal circulation relatively unimpeded (Ikezuki et al., 2002; Milligan et al., 1998; Vandenberg et al., 2007). It is now widely accepted that development is a window of exceptional vulnerability to EDC exposure.

Organization of Reproductive Neuroendocrine Circuits

Go to:

During early development (gestation in humans, gestation and early neonatal life in rodents), the neuroendocrine feedback loops which regulate sex-specific reproductive physiology and behavior are sexually differentiated and organized. The coordination of physiological events and appropriate behavioral responses to them is paramount to reproductive fitness and the stability of a population. For example, the reproductive fitness of an ovulating female that fails to mate or nurse her young is just as diminished as the reproductive fitness of a female that fails to ovulate. Similarly, a male who responds to a soliciting female with aggression rather than courtship has a significantly decreased chance of passing along his genome even if he is

physiologically capable of doing so. Thus, populations can be at risk when the integration of behavioral responses to physiological and environmental cues is disrupted. The organization of the neuroendocrine circuits that coordinate sex-specific physiology and behavior is orchestrated largely by steroid hormones, particularly estrogen, during distinct critical periods in embryonic and postnatal development (Cooke et al., 1998; Gorski, 1985; Simerly, 1998, 2002). The organization of these circuits appears to be particularly vulnerable to disruption by EDCs.

Maturation and function of the vertebrate reproductive system is coordinated by the hypothalamic-pituitary-gonadal (HPG) axis. This system encompasses a complex network of neuronal signaling pathways that enable the regulation of gonadotropin secretion by steroid hormones (Elkind-Hirsch et al., 1981; Gorski et al., 1975). The neural components of the HPG axis span multiple brain areas, primarily the hypothalamus, a diencephalic region important for coordination of many neuroendocrine functions including hunger, thirst, circadian cycles, emotion, body temperature and stress in addition to reproduction. It is responsive to a litany of external signals including day length, hormones, olfactory cues, and glucose levels, among others. Contained within the hypothalamus are discrete populations of neurons that regulate gonadotropin releasing hormone (GnRH) secretion. The network of regulatory inputs from neuronal and glial cells in the brain projecting to these neurons is sexually differentiated by endogenous gonadal hormones (primarily estradiol in rodents but perhaps both estrogens and androgens in humans) through a series of gestational, pre- and perinatal critical periods (Cooke et al., 1998; Gorski, 1985; Simerly, 1998, 2002). In the post-pubertal animal, GnRH release is regulated through feedback effects of gonadal steroids. In both males and females, GnRH secretion is suppressed by steroid negative feedback, the signal for which is thought to arise from the arcuate nucleus (ARC) of the hypothalamus (Kauffman et al., 2007b; Tena-Sempere, 2006). In females, however, GnRH release is augmented once per cycle by estrogens. This positive feedback potentiates the surge in GnRH and, subsequently, luteinizing hormone (LH) that precedes ovulation (Clarke and Pompolo, 2005). This process is now thought to be mediated within the anterior hypothalamus (Adachi et al., 2007; Kauffman et al., 2007b; Tena-Sempere, 2006) and the sex specific organization of this system can be manipulated, inducing long term consequences. For example, it is well established that the administration of steroid hormones, including androgens or estrogens, during the neonatal critical period can masculinize the female rodent brain while castration can effectively prevent defeminization of the male rodent brain (Bakker and Baum, 2008; Baum, 1979; Simerly, 2002). Thus, in males castrated as neonates, the potential for estrogen to evoke a GnRH surge is preserved while, conversely, in females neonatally exposed to estrogens, this capacity is diminished or lost.

The neuroendocrine pathways regulating reproductive behavior are similarly organized and affected by steroid hormones. In rats, lordosis is a reflexive receptive posture made by the female in response to male mounting and is a hallmark indication of sexual receptivity. Whereas circulating estrogens play an essential role in stimulating lordosis in females (Davidson and Bloch, 1969; Lisk, 1969; Komisaruk and Diakow, 1973; Pfaff, 1999; Pfaff and Sakuma, 1979), males rarely display lordosis behavior even after estrogen administration in adulthood (Yamanouchi and Aria, 1976). However, neonatal steroid hormone manipulation can retain in the capacity to evoke lordosis in males, and suppress proceptive behavioral displays by females, indicating that sex-specific behaviors are also organized by steroid hormones in the perinatal period (Gerall, 1967; Gorski, 1963, 1985; Grady et al., 1965; Sodersten, 1978; Patisaul et al., 2009a; Whalen and Nadler, 1963; Whales et al., 1986). Thus, aberrant organization of sex specific neuroendocrine circuits can have profound and permanent effects on sex specific reproductive physiology and behavior. Consequently, the organization of neuroendocrine pathways is considered to be particularly vulnerable to endocrine disruption. Most examples detailed within this review result from endocrine disruption of the neuroendocrine system.

Low Dose Effects: Health Risk or Hype?

Go to:

It is now evident that exposure to endocrine disruptors has the potential to adversely affect reproductive physiology and behavior in vertebrates. The critical question now is if wildlife and human health is at risk from chronic exposure to low doses of these compounds, either alone or in mixtures. DDT was applied liberally and

has a long half life, thus affected wildlife populations had relatively high exposure levels. Moreover, DES has a binding affinity for ERα and ERβ that is roughly equivalent to estradiol and is therefore a potent estrogen agonist (Korach et al., 1978; Sadler et al., 1998). Most endocrine disruptors exist at far lower levels in the environment and have binding affinities 100–10,000 times lower than either estradiol or DES. In some cases, however, blood levels of these compounds can be several fold higher than endogenous estrogen levels and suspected endocrine disruptors are almost always present in both the environment and in bodily fluids as mixtures. Although DDT and many other EDCs are classified as "weak estrogens" the precise mechanisms through which they interact with the vertebrate neuroendocrine system and the exposure levels that produce substantive effects are still largely undetermined. For example, DDT (and its metabolites) is now appreciated to act as both an estrogen agonist and an androgen antagonist highlighting the potential for compounds to have multiple mechanisms of action. Direct binding to steroid receptors is only one mode of action. Endocrine disruption also includes interference with the biosynthesis, transport, or metabolism of hormones or the disruption of the recruitment of binding proteins or transcription factors. Another key issue that remains poorly understood is how these compounds behave within mixtures. Wildlife and human populations are exposed to dozens if not hundreds of compounds simultaneously yet very little is known about how these compounds interact either *in vitro* or *in vivo*.

For simplicity, to address the question of whether or not endocrine disruptors have the potential to affect reproductive physiology and behavior at low doses, this review will focus on three compounds currently gaining wide scientific and public attention: genistein, bisphenol-a (BPA) and the phthalates. genistein and BPA are classified as weak estrogen agonists and thus presumably have similar mechanisms of action (Figure 1). However, genistein, because it is produced by plants and found in soy foods, is widely perceived as healthful while BPA, a component of polycarbonate plastics and epoxy resins, is commercially produced and was recently classified as a toxin in Canada (April, 2008). These attitudes are incongruous if the two compounds indeed elicit similar effects and therefore warrant further comparative investigation. The phthalates are found in a wide variety of products including cosmetics, pharmaceuticals, toys and medical devices. They are classified as androgen antagonists and their use was recently restricted in the US.

Genistein and Female Reproductive Physiology

Go to:

The endocrine disrupting potential of phytoestrogens was first noticed in Australia in the 1940s when abnormally high rates of infertility, abortion, and reproductive abnormalities in newborn lambs were observed in ewes grazing on clover rich pastures (Bennetts and Underwood, 1951; Bennetts et al., 1946). It was ultimately determined that the steroid-like flavonoid phytoestrogens, most notably coumestrol, were responsible for the observed effects (Adams, 1995a,b; Braden et al., 1967). Decades later, a singular case of infertility and liver disease in captive cheetahs placed on a soy-based diet was ultimately attributed to the same class of compounds (Setchell et al., 1987). These incidents are reminiscent of the DDT story in wild bird populations, and have raised questions regarding the potential risk flavonoid phytoestrogens might pose to humans.

The two major classes of phytoestrogens are the lignans and the isoflavonoids. Lignans are minor components of cell walls and the highest concentrations are found in flaxseed (linseed) products, pumpkin seeds, green tea, coffee, and other fiber-rich foods (Axelson et al., 1982; Kuhnle et al., 2008; Mazur and Adlercreutz, 2000; Penalvo et al., 2008; Thompson et al., 1991, 2006). The isoflavonoids are most prevalent in legumes, especially soybeans and soy-based foods including soy infant formula, tofu and soy milk, but detectable levels also occur in fruits, vegetables, whole grains, and even some alcoholic beverages (Adlercreutz and Mazur, 1997; Fletcher, 2003; Franke et al., 1998a; Lapcik et al., 1998; Reinli and Block, 1996; Setchell et al., 1998). Dietary supplements containing high levels of isoflavonoid phytoestrogens are now widely available (Setchell et al., 2001). Of the many isoflavonoids found in soy, the most intensely scrutinized are genistein and daidzein.

Considerable attention is now being paid to the potential endocrine disrupting properties of isoflavonoids because soy consumption is widely promoted as being healthful and has been associated with reduced risk of

cardiovascular disease and hormone dependent cancers (Adlercreutz and Mazur, 1997; Clarkson, 2002; Demonty et al., 2003; Peeters et al., 2003). In 1999, the US Food and Drug Administration (FDA) approved the health claim that daily consumption of 25 g of soy protein can reduce the risk of coronary artery disease (Food and Drug Administration, 1999). Soy consumption is increasing among all age groups, especially infants and children (Cao et al., 2009; Setchell, 2001; Strom et al., 2001). Genistein and other phytoestrogens readily cross the placenta indicating that fetal exposure is also potentially consequential (Todaka et al., 2005). Total isoflavone content in soy infant formula varies but is consistently high among soy foods, averaging near 40 μg total isoflavones per gram of formula (Franke et al., 1998b; Johns et al., 2003; Setchell and Welsh, 1987; Setchell et al., 1997). This translates to a daily intake of approximately 6–9 mg/kg body weight per day, an amount, when adjusted for body weight, which is four to seven times higher than the amount consumed by adults on a traditional soy-based Asian diet or meeting the FDA guidelines (Barnes, 1995) and considerably higher than any synthetic EDC.

So is there cause for concern? The sheep and cheetah cases are disquietingly similar to the bird and other wildlife studies of the 1970s which ultimately identified the endocrine disrupting properties of DDT. But even today the question of whether or not DDT can impact human health is controversial, and such is the case with soy phytoestrogens. Is there any reasonably good evidence that phytoestrogens can have long term adverse health effects in humans following developmental exposure? A pair of studies on Puerto Rican girls associated neonatal phytoestrogen exposure with advanced pubertal onset, but a number of confounding factors including the use of potent estrogens in meat production, make the data problematic and difficult to interpret (Freni-Titulaer et al., 1986; Schoental, 1983). A more recent, retrospective cohort study found that young women fed soy-based infant formula as part of a controlled, University of Iowa feeding study reported longer menstrual bleeding and menstrual discomfort than those who were fed a non-soy based formula as babies (Strom et al., 2001). Beyond these epidemiology studies, very little is known about how exposure to soy phytoestrogens, either in the womb or in infancy, impacts female reproductive health or behavior in humans.

Data from animal research is more abundant. Neonatal exposure to genistein advances pubertal onset, increases the length of the estrous (menstrual) cycle and hastens the onset of persistent estrus in rodents. Female mice treated with 0.5–50 mg/kg genistein for only the first 5 days of life give birth to fewer live pups over time compared to untreated control animals, with fertility most strongly impacted at the highest dose (Jefferson et al., 2005). This acceleration of reproductive senescence could result from disruption anywhere within the HPG axis including the ovary and brain. Detailed work in mice by Jefferson and colleagues has revealed that genistein can interfere with ovarian differentiation resulting in ovarian malformations indicative of impaired fecundity such as multi-oocyte follicles, and attenuated oocyte cell death (Jefferson et al., 2002, 2006, 2007). Ovarian defects, including the absence of corpora lutea, the presence of large antral-like follicles with degenerating or no oocytes and numerous ovarian cysts have also been observed following neonatal genistein exposure in rats (Kouki et al., 2003) (Figure 2).

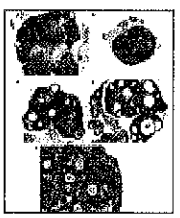

Figure 2

Frequently observed ovarian malformations in rats following neonatal exposure to endocrine disruptors. (A) An ovary from an unexposed adult female contains follicles at all stages of folliculogenesis and numerous corpora lutea (CLs), indicative of successful **...**

Recent studies in our laboratory have found that the organization of sexually differentiated neural pathways within the hypothalamus is also vulnerable to neonatal endocrine disruption by genistein. We determined that advanced vaginal opening and abnormal estrous cyclicity, induced by neonatal exposure to 10 mg/kg genistein, is accompanied by an impaired ability to stimulate GnRH neuronal activity (as measured by the immunoreactivity of both of GnRH and Fos) following ovariectomy and hormone priming (Bateman and Patisaul, 2008) (Figure 3). This observation indicates that neonatal genistein exposure has a masculinizing

effect on the female HPG axis. Although GnRH neurons express ERβ throughout development (Herbison and Pape, 2001; Hrabovszky et al., 2000, 2001) and thus could potentially respond to neonatal genistein directly, it is generally accepted that hormonal and other environmental signals are largely conveyed to GnRH neurons from other estrogen-responsive neurons clustered in different regions of the hypothalamus. In rodents, the two most significant regions appear to be the anterior ventral periventricular (AVPV) and arcuate (ARC) nuclei (Gu and Simerly, 1997; Polston et al., 2004; Polston and Simerly, 2006; Shughrue et al., 1997; Simerly et al., 1990) both of which contain sexually dimorphic populations of neurons that express the KiSS-1 gene. This gene codes for a family of peptides called kisspeptins (previously called metastins), and rapidly emerging evidence indicates that kisspeptin neurons are essential for coordinating pubertal onset and steroid feedback on GnRH neurons in many species, including humans (Kauffman et al., 2007a; Navarro et al., 2004; Smith et al., 2006a,b). AVPV kisspeptin neurons are more numerous in females than males and are thought to be essential for steroid positive feedback and the initiation of the preovulatory GnRH surge (Clarkson et al., 2008; Gottsch et al., 2004; Irwig et al., 2004; Kauffman et al., 2007a, Roa et al., 2006; Smith et al., 2006b). In contrast, KiSS mRNA expression in the ARC is not thought to be sexually dimorphic and appears to be important for the regulation of steroid negative feedback (Kauffman et al., 2007a). We have now shown that neonatal exposure to 10 mg/kg genistein can significantly decrease the density of neuronal fibers immunolabeled for kisspeptin in the AVPV but not the ARC of female rats (Bateman and Patisaul, 2008; Patisaul et al., 2009b) indicating that disrupted organization of the kisspeptin signaling pathways may be a novel yet fundamental mechanism by which a suite of reproductive abnormalities are induced including disrupted timing of pubertal onset, irregular estrous cycles and premature anovulation.

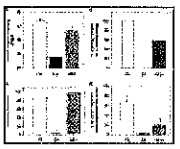

Figure 3

Effects of neonatal exposure to genistein (GEN, 10 mg/kg bw) compared to an oil based vehicle (OIL), and estradiol benzoate (EB, 50 μg) on reproductive physiology and behavior in female rats. (A) GEN significantly advanced the ...

Interestingly, animals exposed to genistein neonatally remained capable of displaying lordosis when ovariectomized, primed by the sequential administration of estradiol benzoate and progesterone, and paired with vigorous males (Figure 3). Lordosis behavior is also retained by female rats neonatally exposed to agonists selective for either ERα or ERβ but not estradiol benzoate suggesting that agonism of both ER subtypes may be needed to fully defeminize the behavior (Patchev et al., 2004; Patisaul et al., 2009a). It may also take a longer exposure or higher doses, a possibility which illustrates the important concepts of dose and timing when considering the potential effects of EDCs. Further complicating the issue is a report that selective agonism of ERβ results in a statistically significant reduction, but not elimination, of lordosis behavior in mice (Kudwa et al., 2006). This could be a species difference in sensitivity or the relative role ERβ plays in the organization of the neuroendocrine pathways that mediate the lordosis response. Further work will be needed to clarify this issue.

The mechanisms by which genistein and the other phytoestrogens influence sex-specific physiology are likely diverse. Genistein has a higher relative binding affinity for both ERα and ERβ *in vitro* than most other EDCs and a higher binding affinity for ERβ that for ERα (Kuiper et al., 1998). Genistein is also a potent inhibitor of tyrosine protein kinases (Boutin, 1994; Piontek et al., 1993), which catalyze phosphorylation of their own tyrosine residues and those of other proteins, including growth factors involved in tumor cell proliferation. In addition, genistein can also inhibit DNA topoisomerases I and II, enzymes essential for DNA replication (Kurzer and Xu, 1997; Okura et al., 1988) and may also work through epigenetic mechanisms involving both hyper and hypomethylation (Dolinoy et al., 2006; Tang et al., 2008). A further complication is the observation that effects of genistein administration at low doses are compounded when co-administered with other EDCs (Kurzer and Xu, 1997; You et al., 2002). Mixture effects of phytoestrogens, as with most other EDCs, are generally unappreciated.

Bisphenol-A (BPA): A Dynamic and ongoing Controversy

Go to:

BPA is somewhat unique among EDCs because, in the early twentieth century, it was being developed as a possible synthetic estrogen (a pursuit which was abandoned following the synthesis of DES) (Dodds and Lawson, 1936). Since the 1950s, it has been used primarily in the production of polycarbonate plastic products to improve clarity and increase resilience. It is also a component of epoxy resins used to line the interior of metal cans such as soda and soup cans. Human exposure to BPA occurs through everyday use of these items because it can migrate from the container into the contents, especially when heated (Brede et al., 2003). The United States Centers for Disease Control recently estimated that nearly all Americans have detectable levels of BPA in their bodies, with children having higher levels than adults (Calafat et al., 2005, 2008). Infants in neonatal intensive care units have particularly high exposure to BPA, presumably from its use in medical devices and from the migration of BPA into infant formula from the container (Calafat et al., 2009). In addition, newborns can be exposed through lactational transfer (Tsutsumi, 2005) and relatively high levels in umbilical cord blood and fetal serum (compared to maternal blood levels) indicate that BPA fails to bind α-fetoprotein and can expose the developing fetus (Ikezuki et al., 2002; Vandenberg et al., 2007). Exposure to BPA is not limited to humans, and effects on wildlife from contaminated water supplies have been documented in both males and females of multiple species (Crain et al., 2007; Maffini et al., 2006; vom Saal et al., 2007).

Although it was once thought that BPA could function as a synthetic estrogen, BPA was not considered to pose a significant threat to either wildlife or human populations because its binding affinity for the primary forms of the estrogen receptor (ERα and ERβ) is approximately 10,000-fold weaker than that of estradiol or DES (Gould et al., 1998; Kuiper et al., 1998). Yet, numerous studies from multiple laboratories have shown that BPA can impact reproductive physiology and behavior in rodents at doses even lower than the current reference or "safe" exposure limit for humans of 50 μg/kg body weight per day (Vandenberg et al., 2009). The mechanism (s) of low dose activity remains poorly understood but has long been hypothesized to be most potent in hormone sensitive organs and brain regions when endogenous estrogens are low or absent (vom Saal and Moyer, 1985). Thus, like DES, at different life stages BPA may have profoundly different effects on the same brain region or organ. It may also act through nonclassical estrogen pathways such as those mediated via membrane ERs, GPR30 or the newly discovered estrogen-related receptor –γ (ERR-γ) (Matsushima et al., 2007; Thomas and Dong, 2006; Watson et al., 2007).

Despite the controversy surrounding the mechanism by which low dose exposure to BPA could affect reproductive physiology, the evidence for widespread effects in animal models is increasing. Effects of perinatal exposure observed at the 50 μg/kg body weight dose or lower include advanced vaginal opening and the hastened onset of first estrus indicating advanced puberty (Adewale et al., (in press); Honma et al., 2002). Ovarian malformations including increased number of blood-filled ovarian bursae, indicative of advanced reproductive aging, abnormal numbers of antral follicles, aneuploidy and decreased corpora lutea (Figure 2) have also been observed by us and others (Adewale et al., (in press); Markey et al., 2003; Susiarjo et al., 2007). BPA can also induce apoptosis and cell arrest in cultured ovarian granulosa cells (Xu et al., 2002). Prolonged, irregular estrus cycles are also frequently observed by us and others following perinatal exposure to low doses suggesting compromised fertility (Adewale et al., (in press); Markey et al., 2003). Mammary gland development appears to be particularly sensitive to BPA at multiple points in the life span including embryonic development, the perinatal period, puberty and adulthood (Markey et al., 2001, 2003; Wadia et al., 2007), effects which may be mediated by an ERα-dependent mechanism (Recchia et al., 2004; Vivacqua et al., 2003). Defects include intraductal hyperplasias, increased sensitivity to endogenous estradiol and the development of neoplastic lesions (Durando et al., 2007).

Like genistein, BPA may also compromise sexual differentiation in the brain. We determined that exposure to 500 μg BPA for only 2 days, beginning the day after birth, feminized the number of dopaminergic neurons in the pubertal male rat AVPV (Patisaul et al., 2006) but not the overall size of the AVPV (Patisaul et al., 2007). Another laboratory subsequently reported decreased numbers of dopaminergic neurons in the AVPV of female

mice exposed from gestation through lactation to lower, environmentally relevant doses (Rubin et al., 2006). Collectively, these studies suggest that each sex may be susceptible to different doses, a hypothesis that is intriguing and merits further exploration as the observed discrepancy could also be due to differences in, route of administration, duration of exposure or species; all of which illustrates the challenge of translating animal data to human risk assessments. Notably, other studies have also reported phenotypic changes in the AVPV after neonatal exposure to EDCs (Gore, 2008). Exposure to Aroclor 1221, a mixture of PCBs, through late gestation and the early neonatal period reduced the number of ERβ-expressing cells in the adult female rat AVPV without markedly affecting volume (Salama et al., 2003). Similarly, exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on gestational day 15 abolished the sexually dimorphic expression of GAD 67 in the rat AVPV on PND 3 (Hays et al., 2002). Although these data support the hypothesis that sexual differentiation of the brain in general, and within the AVPV specifically, may be vulnerable to endocrine disruption, the physiological and functional consequences of these changes has yet to be convincingly established.

Considerable scientific debate remains about whether or not humans are exposed to truly significant levels of BPA. Human serum levels have generally been found to be in the range of 0.2–20 ng/ml (Vandenberg et al., 2007) and urinary levels increase following the consumption of beverages from polycarbonate bottles containing BPA (Carwile et al., in press). Most rodent studies evaluating potential adverse effects of BPA did not measure serum levels in exposed animals making it difficult to determine if the outcomes can be extrapolated to humans. Further complicating the issue is that humans and rodents metabolize BPA differently, however both species, along with non-human primates, exhibit comparable levels of the unconjugated (estrogenically active) form of BPA in fluids and thus perhaps equally susceptible to its effects (reviewed in detail by Vandenberg et al., 2009). Like DDT, the fate of BPA may ultimately be decided by politics and public perception, rather than a regulatory action based on a measured evaluation of the scientific evidence. As this manuscript went to press (May, 2009) Canada declared BPA a "toxin" and Minnesota became the first state to ban the use of BPA in baby bottles and cups. Many other states had similar laws pending. In October of 2008, the Food and Drug Administration reaffirmed its conclusion that BPA use in food containers does not pose a public health threat, a decision that was met with considerable criticism. Not long after, Nalgene and most baby bottle manufacturers announced that they would no longer use BPA in the manufacturing of their products and bottles labeled "BPA-Free" are becoming increasingly common in Wal-Mart and other retail outlets.

Endocrine Disruption of Male Reproductive Physiology by DDT and the Phthalates

Go to:

Endocrine disruption can also happen in males. One of the most notable wildlife cases has been documented by researchers at the University of Florida who discovered numerous genital malformations, poor hatching success, and a sex ratio heavily skewed towards females among alligators living in a polluted central Florida lake. This lake had become heavily contaminated with DDT, difocol, their metabolites, and other pesticides as the result of an industrial spill (Guillette et al., 1994, 1995b; Milnes et al., 2005; Semenza et al., 1997). In mammals and birds, gonadal differentiation (the development of either an ovary or testis) is determined by the sex chromosomes. In non-mammalian vertebrates, including reptiles, incubation temperature during a critical window midway through development, influences which gonad forms and thus the sex of the animal. Guillette and colleagues discovered that exposure to estrogens or estrogenic EDCs can override or interact with this effect of temperature resulting in intersex individuals, males with abnormally low plasma testosterone levels, genital abnormalities, and malformation of the gonads (in both sexes). Exposing turtle or alligator eggs experimentally with the DDT metabolite DDE or estradiol induced similar effects (Bergeron et al., 1994; Crews et al., 1995; Gale et al., 2002; Guillette et al., 1995a) conclusively demonstrating that endocrine disruption of male sex determination and the differentiation of the male reproductive system is possible. Reproductive abnormalities within reptiles living on this lake persist, even more than three decades after the initial spill, demonstrating that defects can impact multiple generations, particularly when the compounds are strongly lipophilic and have long half-lives.

In humans, there is growing evidence for, but considerable debate over, whether human male reproductive health is truly declining, and whether EDCs play any role in the perceived decline. In 1992, Carlsen and colleagues conducted a comprehensive review of the literature on human semen quality. Their systematic analysis of 61 published papers, incorporating data collected from nearly 15,000 men, revealed a statistically significant decline in mean seminal volume and sperm concentration over the last 50 years (Carlsen et al., 1992). This finding was widely publicized in the media and subsequently replicated by other investigators, although there appear to be significant regional differences in the severity of the effect (Swan et al., 2000, 2003). Similarly, the incidence of testicular cancer and congenital abnormalities such as hypospadias and cryptorchidism also appear to be increasing (Adami et al., 1994; SEER, 2003; Sharpe, 2003) signifying a comprehensive decline in male reproductive health over the past 50 years. Doubt over the conclusions from these analyses persist however, because reliance on historical data sets (retrospective studies) restricts the ability to control for differences in data collection methods. With this caveat in mind, one feature of the semen data that stands out is an apparent birth cohort effect, with younger generations having poorer semen quality than older generations (Irvine, 1994). This suggests that insult in fetal life could be responsible for the defects that emerge later. This hypothesis is supported by epidemiological data showing that the occurrence of one disorder, such as low sperm count, is a risk factor for the occurrence of another, such as testicular cancer. This relationship has led a Danish research group to propose that low sperm counts, hypospadias, cryptorchidism and testicular germ cell cancer are interrelated disorders, all of which have their roots in fetal development, comprising a "testicular dysgenesis syndrome" (TDS) (Boisen et al., 2001; Skakkebaek et al., 2001, 2006). The TDS hypothesis proposes that these disorders are all manifestations of disturbed prenatal testicular development resulting from abnormal hormone synthesis or action during reproductive tract development.

Androgens, including testosterone, produced by the fetal testes are essential for the differentiation of the epididymis, vas deferens and the seminal vesicles from the Wolffian ducts. Differentiation of the prostate and external genitalia requires 5α-dihydrotestosterone (DHT), the most potent androgen produced by the testis catalyzed from testosterone by the enzyme 5α-reductase. Failure to produce DHT, or sufficient levels of DHT, can lead to poorly developed or malformed external genitalia. Therefore toxicants that interfere with 5α-reductase or the interaction of DHT with the androgen receptor during development can impair proper development of male genitalia and the prostate. For example, administration of the androgen receptor antagonist, flutamide during male reproductive tract development induces multiple abnormalities of the external genitalia including hypospadias and cryptorchidism in both rats and monkeys (Herman et al., 2000; Mylchreest et al., 1999). Environmental toxicants are also known to interfere with male genital development. One of the earliest animal studies designed to test the hypothesis that chemical agents could interfere with androgen action was conducted in 1950 using chickens. Injection of DDT resulted in markedly undersized testes and inhibited the development of the comb and wattle. It was later determined that DDT and its metabolites function as anti-androgens (as well as estrogen mimics) and compete with endogenous androgens for access to the androgen receptor.

One class of compounds that has recently received considerable attention for potentially contributing to TDS is the phthalates. There are many different kinds of phthalates and the two considered to have the greatest potential to impact male reproduction are dibutyl phthalate (DBP) and diethylhexyl phthalate (DEHP, Figure 1). DBP is used in many personal care products such as lotions, cosmetics, nail polish, and perfume. DEHP is primarily used as a plasticizer in the production of flexible products including vinyl, medical tubing and toys. Infants in neonatal intensive care units have some of the highest urinary phthalate levels observed to date, presumably a result of exposure through medical tubing and devices (Calafat et al., 2004; Weuve et al., 2006). A series of studies conducted in rats in the late 1990s was the first to demonstrate that phthalates could interfere with the ability of testosterone to masculinize the male reproductive tract. Exposure *in utero*, when the genitals are being formed, resulted in a number of genital malformations including hypospadias and hemorrhagic testes (Gray et al., 1999; Wolf et al., 2000). Thus it is plausible that neonatal exposure to phthalates could induce TDS. Interestingly, the phthalates do not produce their effects by antagonizing the

androgen receptor, but rather by interfering with the production of androgens in the fetal testis (David, 2006). Exposure to phthalates during human pregnancy has now been associated with smaller (feminized) anogenital distance in infant boys (Swan et al., 2005). Epidemiological evidence has also positively correlated higher urinary phthalate levels with lower sperm counts and an increased likelihood of sperm with damaged DNA in adult men (Duty et al., 2003; Hauser, 2008; Pant et al., 2008; Wirth et al., 2008). Although it is important to keep in mind that correlation does not prove causation, these newly emerging epidemiology studies are the best evidence to data that phthalates have the potential to affect male reproductive health in humans.

Mixture Effects: When the Quality of Bird Song Constitutes "False Advertising"

Go to:

Intriguingly, exposure to EDCs can also enhance the masculinization of some traits but this many not translate to improved reproductive fitness. A good example of this occurred in song birds exposed to a mixture of compounds including phthalates. In song birds, song production is controlled by discrete neural pathways which develop and operate under the influence of steroid hormones. Estrogens, as well as androgens to some degree, are essential for proper sex specific organization of the song system. According to sexual selection theory, male secondary sex characteristics, including the production of elaborate songs, have evolved as indicators of male quality and in response to female preferences. Thus, modification of the song system by exposure to estrogenic contaminants could affect song quality and, as a result, how attractive exposed males are to potential mates. A recent experiment by Markman and colleagues tested this hypothesis using European starlings (*Sturnus vulgaris*) (Markman et al., 2008). The research group first observed the foraging habits of wild starlings at 20 sewage treatment sites and determined that earthworms were a primary prey species. Subsequent analysis of these earthworms revealed that they were heavily contaminated with 17β-estradiol (E2, from human wastewater), phthalates and BPA. A daily exposure for each compound was then estimated. Wild-caught juvenile birds were then maintained in the laboratory and exposed to either vehicle, 200 ng E2 or the environmentally relevant mixture (200 ng E2, 640 ng phthalates, 80 ng BPA) daily by injecting each preparation into a mealworm. The birds were exposed from October to April, when foraging on sewage beds is common, prior to the onset of the breeding season in the spring. Males exposed to the mixture had significantly enhanced song production including a more complex song repertoire, an increased number of song bouts, and longer songs. This was accompanied by enlargement of an important song nucleus, the HVC, in the males fed the mixture compared to control males. Females preferred the song of males fed the mixture to males fed either the vehicle or the E2 alone.

Enhanced song production, however, in this case appears to be a false indicator of male quality because subsequent analysis found that, even though circulating androgen levels were comparable to controls, the males fed the mixture were immunocompromised. Thus, although EDCs enhanced the masculinization of the song system, by misleading females into choosing less fit males the effect could subsequently decrease the overall fitness of the population.

Interestingly, males fed only E2 did not show enhanced song production or larger song nuclei demonstrating that this component of the mixture was insufficient on its own to influence this estrogen sensitive behavior. This finding emphasizes the concern that although some estrogenic compounds may not produce effects at low levels on their own, they may ultimately contribute to disruption when contained within a mixture of compounds with similar mechanisms of action. This concern is potentially considerable for a number of reasons. Perhaps one of the most worrisome is that, because most laboratory animal diets are derived from soy and therefore contain relatively high levels of genistein and other phytoestrogens (actual amounts vary from batch to batch) this background of EDC exposure could impact endocrine disruption research (Brown and Setchell, 2001; Degen et al., 2002). Could this be a significant potential confound? A research group at Washington State University recently reported that they could not replicate previously published BPA effects in the mouse ovary. They ultimately concluded that effects were only observed in mice maintained on soy-rich diets leading the authors to hypothesize that diet, along with methodological differences, could explain why the literature surrounding "low dose" effects of BPA is fragmented and inconsistent (Muhlhauser et al., 2009). The

concept of mixture effects is an evolving area of endocrine disruption research, the results of which could have profound implications for other disciplines including neuroendocrinology and behavioral biology.

Transgenerational Effects and the Emerging Field of Epigenetics

Go to:

It is now becoming evident that the effects of EDC exposure are not necessarily limited to the exposed individual. Many of these compounds are now recognized to have transgenerational effects and in some cases the effects within subsequent generations are more profound than those seen in the first generation (Jirtle and Skinner, 2007; Steinberg et al., 2008). For example, there is emerging concern that the children of DES daughters (referred to as DES granddaughters) might also experience reproductive problems. For these girls, their exposure occurred when they were germ cells in their mother's developing ovary, within the womb of their grandmother. If true, it would be the first instance in humans which conclusively demonstrates that persistent, generational effects can result from an *in utero* exposure to a potent estrogen (Newbold et al., 1998). This concern for DES granddaughters arose from data obtained in laboratory animal studies which indicated that the offspring of females exposed *in utero* were more likely to develop reproductive tract lesions than unexposed control animals (Newbold et al., 1998, 2000). Other studies have produced evidence that DES effects across generations can be transmitted through the paternal line as well (Walker and Haven, 1997). So far, there is not enough human data to indicate a trend for deleterious effects in DES granddaughters, largely because this cohort is so young. Continued monitoring of these women as they age will ultimately be required to determine if there are transgenerational effects of prenatal DES exposure in humans.

The precise mechanisms through which endocrine disrupting effects transmit to subsequent generations are not well understood but emerging evidence indicates alteration of chromosomal structure or other epigenetic mechanisms might be the primary method. Epigenetic inheritance involves changes in gene expression patterns without changes in gene sequence. Such effects include DNA methylation and histone modifications, among others. In most cases, methylation of gene promoter regions abrogates gene transcription while acetylation of the histone tail enhances it (Dolinoy et al., 2007; Gore, 2008; Ho and Tang, 2007). These processes can be influenced by environmental factors and if these modifications occur within the germ cell lines then transmission to subsequent generations is possible (Giusti et al., 1995; Gore, 2008; Jirtle and Skinner, 2007). This newly discovered and evolving area of research has once again transformed the field of toxicology and introduced a novel method by which endocrine disruptors and other toxicants can affect vertebrate physiology and behavior.

A well characterized animal model demonstrating the potential for epigenetic modifications to impact future generations involves the manipulation of the murine agouti gene in a specialized mouse strain (Duhl et al., 1994; Vrieling et al., 1994). In this strain, the degree of methylation on an inserted transposable element can vary dramatically and correlates with a wide distribution in coat color that ranges from yellow (unmethylated) to brown (methylated) as well as the occurrence of diabetes, obesity and tumorigenesis. Maternal dietary supplementation of methyl-donors (folic acid, vitamin B_{12}, choline and betaine) during pregnancy can shift the coat color of offspring towards the brown pseudoagouti phenotype (Duhl et al., 1994; Waterland and Jirtle, 2003; Wolff et al., 1998) demonstrating the effect maternal diet can have on offspring adult phenotypes. Even more intriguing is the observation that maternal exposure to genistein induces hypermethylation, again shifting the coat color of the offspring to brown (Dolinoy et al., 2006), but unlike other methyl donors, genistein protected these offspring from the obesity normally associated with the darker color. These results illustrate the potential for genistein and perhaps other EDCs to epigenetically alter the phenotype of subsequent generations through *in utero* exposure. Other compounds, including polychlorinated biphenyls (PCBs) (Aubrecht et al., 1995; Schiestl et al., 1997) and the fungicide vinclozolin (Anway et al., 2006; Gore, 2008) have also been shown to produce transgenerational effects, perhaps through epigenetic mechanisms.

When a pregnant animal is exposed to an EDC, it is important to keep in mind that the mother (F_0), the embryo (F_1) and the F_2 generation (as germ cells) are all directly exposed. While the majority of studies concerning transgenerational epigenetic effects of EDCs have not been carried past the F_2 generation it is

important to note that, to rule out direct exposure effects, the F_3 generation should also be examined for abnormalities. It is also critical to appreciate that epigenetic effects can also occur outside of the germ line. Thus these mechanisms may underlie many observed EDC effects and could explain how compounds which are only weakly estrogenic, like BPA, can produce appreciable, lasting results at such low levels (Jirtle and Skinner, 2007). The ability of most estrogenic EDCs, such as DES, genistein and BPA, to pass from mother to offspring through placental blood flow or lactational transfer makes the possibility for epigenetic, transgenerational effects likely (Crews and McLachlan, 2006; Franke and Custer, 1996; Mably et al., 1992; Newbold, 2006; Ruden et al., 2005; Sun et al., 2004; Wisniewski et al., 2003). Although the specific mechanisms underlying the observed transgenerational effects of DES in animals have proven difficult to elucidate, emerging research implicates epigenetic modifications as a significant component (Gore, 2008; Li et al., 2003; Newbold, 2006). Newbold and colleagues have recently shown that alterations in gene methylation patterns of estrogen-responsive genes following DES exposure can be passed on to the next generation (Li et al., 1997, 2003; Newbold, 2006). Studies exploring how epigenetics might explain the "fetal origins of adult disease" are currently underway in DES granddaughters, but results will emerge slowly because of the relatively young age of the subjects. Although clear indicators of illness occurred early in some individuals, most carcinogenic and reproductive tract abnormalities in DES daughters did not occur until at least middle age and it will be another decade before most of the DES granddaughters reach that age as well. Thus, many transgenerational effects in these individuals have likely not yet emerged (Giusti et al., 1995; Rubin, 2007).

Conclusions

Go to:

While it appears clear, in both animals and humans, that exposure to EDCs can have adverse effects on reproductive physiology and behavior, controversies surrounding this topic remain. Importantly, recognition of the prevalence of these compounds in the environment and their potential to adversely affect both wildlife and human populations is increasing among scientists, policy makers, and the general public. Further efforts to understand the mechanisms underlying EDC effects, particularly those seen at environmentally relevant doses by compounds with low hormonal potency, are necessary to adequately develop a public health strategy for preventing or combating their effects. The ability of these compounds to permanently affect the epigenome could be potentially catastrophic to the welfare of future generations and requires further attention by both toxicologists and endocrinologists. While research surrounding this topic is not conclusive, particularly in humans, there is certainly sufficient evidence to warrant concern about potential long term effects in both wildlife and humans. Obtaining absolute proof of endocrine disruption by BPA, phthalates, and other compounds with weak hormonal activity in humans is likely impossible because it would obviously be unethical to conduct a double-blind study where one group is exposed to a suspected toxicant. Research in animals, however, is robust and indicates that disruption of sex specific behavior, neuroendocrine circuitry and physiology is possible and, in some cases, transgenerational.

Unfortunately, it is extraordinarily difficult for individuals to make informed choices about how to reduce their potential exposure because chemicals in the US are not routinely screened or tested for endocrine disrupting properties. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was formed by Congress in 1996 to make specific recommendations to the EPA about how to test and screen compounds for endocrine disrupting properties, but progress has been frustratingly slow. A list of compounds to be screened was not compiled until April of 2009 and only 67 chemicals were included, a tiny fraction of the thousands of compounds now suspected of having endocrine disrupting properties. Moreover, it is often impossible to determine which plastics, cosmetics, toys, or other household items contain any of these compounds so consumers have no adequate way to avoid them if desired. The thought that the mixture of chemicals a pregnant woman is exposed to during her pregnancy could affect not only her daughter's fecundity but also her granddaughter's is alarming and a major reason why the topic of endocrine disruption continues to receive global attention by scientists and the general public.

Conflict of Interest Statement

Go to:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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Footnotes

Go to:

[1] http://www.epa.gov/endocrine/Project.html

[2] http://apps.who.int/malaria/wmr2008/malaria2008.pdf

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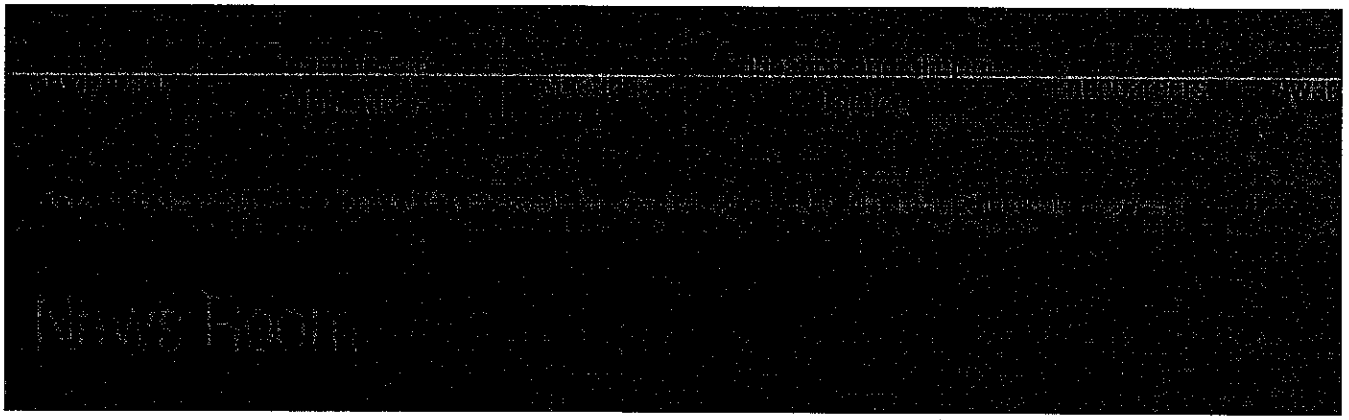

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Experts Say Protocols for Ider -Disrupting Chemicals Inadeq

Endocrine Society issues Statement of Principles on endocrin health protection

Chevy Chase, MD— In a Statement of Principles unveiled today, T streamlined definition for endocrine-disrupting chemicals (EDCs) a strengthen the ability of current screening programs to identify EDC

An endocrine-disrupting chemical (EDC) is a chemical or mixture o interfere with any aspect of hormone action. The Endocrine Society 2009 provided an exhaustive summary of the scientific background EDC exposures to humans and wildlife.

"Because of the interest and expertise of our members, The Endoc help inform the ongoing debate about the health effects of endocrir PhD, of the University of Massachusetts and lead author of the stat key issues related to identifying EDCs and protecting humans and

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Environ Health Perspect; DOI:10.1289/ehp.121-A298

Uncertain Inheritance: Transgenerational Effects of Environmental Exposures

Charles W. Schmidt, MS, an award-winning science writer from Portland, ME, has written for *Discover Magazine*, *Science*, and *Nature Medicine*.

About This Article open

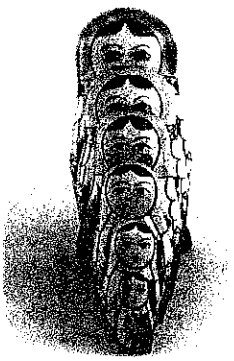
PDF Version (3 MB)

Andrea Cupp made a serendipitous discovery when she was a postdoctoral fellow at Washington State University: While investigating how chemicals affect sex determination in embryonic animals, she bred the offspring of pregnant rats that had been dosed with an insecticide called methoxyclor. When the males from that litter grew into adults, they had decreased sperm counts and higher rates of infertility. Cupp had seen these same abnormalities in the animals' fathers, which had been exposed to methoxyclor in the womb. But this latest generation hadn't been exposed that way, which suggested that methoxyclor's toxic effects had carried over generations. "At first I couldn't believe it," says Cupp's advisor, Michael Skinner, a biochemist and Washington State professor. "But then we repeated the breeding experiments and found that the results held up."

Skinner and Cupp, who is now a professor at the University of Nebraska–Lincoln, published their findings in 2005.[1] Since that paper—which showed that reproductive effects not just from methoxyclor but also from the fungicide vinclozolin persisted for at least four generations—the number of published articles reporting similar transgenerational findings has increased steadily. "In the last year and half there's been an explosion in studies showing transgenerational effects from exposure to a wide array of environmental stressors," says Lisa Chadwick, a program administrator at the National Institute of Environmental Health Sciences (NIEHS). "This is a field that's really starting to take off."

According to Chadwick, the new findings compel a reevaluation of how scientists perceive environmental health threats. "We have to think more long-term about the effects of chemicals that we're exposed to every day," she says. "This new research suggests they could have consequences not just for our own health and for that of our children, but also for the health of generations to come."

The NIEHS recently issued requests for applications totaling $3 million for research on transgenerational effects in mammals.[2] Chadwick says funded studies will address two fundamental data needs, one pertaining to potential transgenerational mechanisms and another to the number of chemicals thought to exert these effects. These studies will extend to what's known as the F_3 generation—the great-grandchildren of the originally exposed animal. That's because chemicals given to pregnant females (the F_0 generation) interact not only with the fetal offspring (the F_1 generation) but also the germ cells developing within those offspring, which mature into the sperm and eggs that give rise to the F_2 generation.



Glossary

Epigenetic—Refers to alterations in gene expression potential that can be passed down through generations.

F_0, F_1, etc.—Shorthand used to distinguish successive generations from one another. "F" stands for "filial generation."

Germ line—The genetic lineage of germ cells (egg and sperm progenitors) that passes down through generations of individuals.

Thus, the F_3 animals are the first generation to be totally unexposed to the original agent. Effects that extend to the F_2 generation are known as "multigenerational," whereas those that extend to the F_3 generation are known as "transgenerational."[1]

Transgenerational effects have now been reported for chemicals including permethrin, DEET, bisphenol A, certain phthalates, dioxin, jet fuel mixtures, nicotine, and tributyltin, among others. Most of these findings come from rodent studies.[4,5,6,7] But preliminary evidence that chemical effects can carry over generations in humans is also emerging, although no F_3 data have been published yet. Given the challenges of tracking effects over multiple human lifespans, the evidence is more difficult to interpret, particularly with respect to potential mechanisms, says Tessa Roseboom, a professor of early development and health at the Academic Medical Center in Amsterdam, the Netherlands. Still, some reports have linked nutritional deficiencies from famine and exposure to diethylstilbestrol (DES)—a nonsteroidal estrogen used to protect against miscarriage from the 1940s to the 1970s—to effects that persist among the grandchildren of exposed women.[8,9,10,11,12,13]

Imprinted gene—A gene whose expression is determined by whether it comes from the mother or the father.

Marks (or Tags)—Molecules that attach to DNA and influence gene expression.

Methylation—Modification of DNA by the addition of a type of molecule known as a methyl group.

Multigenerational—Refers to effects that extend to the F_2 (grandchild) generation.

NOAEL—The highest dose that produces no adverse effects in exposed animals during a toxicology study.

Transgenerational—Refers to effects that extend to the F_3 (great-grandchild) generation.

Foundations in Animal Data

The way in which environmental exposures cause transgenerational effects is unclear. According to Chadwick, current hypotheses lean toward epigenetic inheritance patterns, which involve chemical modifications to the DNA rather than mutations of the DNA sequence itself. Scientists debate the precise definition of "epigenetics," but Robert Waterland, an associate professor of pediatrics and genetics at Baylor College of Medicine, suggests the best definition was published in *Nature Genetics* 10 years ago: "The study of stable alterations in gene expression potential that arise during development and cell proliferation."[14]

Epigenetic modifications can take a few different forms—molecules known as methyl groups can attach to DNA itself, or methyl or acetyl groups can attach to the histone proteins that surround DNA. These attached molecules, also known as "marks" or "tags," influence gene expression and thereby determine the specialized function of every cell in the organism.

Epigenetic marks carried over from the parents are typically wiped clean during molecular programming events that happen early in embryonic development. Shortly after fertilization, explains Dana Dolinoy, an assistant professor at the University of Michigan School of Public Health, a wave of DNA demethylation leaves the embryo with a fresh genomic slate with the exception of certain imprinted genes, such as insulin-like growth factor 2 (*IGF2*), which remain methylated. Later, cells in the developing embryo are remethylated as they develop into the somatic cells that make up different organs and tissues in the body. Germ cells, meanwhile, undergo their own wave of demethylation and remethylation programming events, which are specific to the sex of the developing embryo.

Researchers have found that transgenerational effects can result from chemical dosing at precise windows in fetal development—specifically, at the time of sex determination, which occurs around embryonic days 10.5–12.5 for mice and embryonic days 41–44 for humans, according to Duke University cell biology professor Blanche Capel. Observable effects in the F_3 generation are thought to result from changes to the germ line, which is the succession of germ cell DNA that passes from one generation to the next. Skinner and other researchers have identified DNA methylation changes in F_3-generation sperm that appear to underlie transgenerational effects seen in F_3 animals.[14]

Researchers emphasize that much of the evidence so far in the field is observational, meaning the biological mechanisms remain unknown. Dolinoy says scientific opinions lean heavily toward epigenetic pathways. "That seems to be where the whole field is headed," she says.

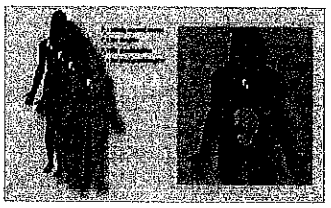
According to Chadwick, Skinner's laboratory remains a nexus for transgenerational studies in chemically exposed animals. In his more recent work, Skinner has shown that insecticides, phthalates, dioxin, and jet fuel, when given to gestating rats during periods of embryonic programming, promote early-onset puberty in female offspring and decreased sperm counts in males, out to the F_3 generation.[4] "We mapped DNA methylation in germ cells and found that each compound induces a unique epigenetic signature," Skinner says. "But it's also possible that other epigenetic mechanisms play a role."

Meanwhile, several other groups are studying transgenerational changes in animals. In one study, Kwan Hee Kim, a professor of molecular biosciences at Washington State, exposed pregnant mice to di-(2-ethylhexyl) phthalate (DEHP) on embryonic days 7–14.[7] Kim observed decreased sperm counts and sperm motility in male offspring out to the F_4 generation. Importantly, she also observed an 80% reduction in spermatogonial stem cell regeneration. Consequently, she says, "As the animals aged, their ability to make new sperm decreased dramatically."

Kim implicates DNA methylation as a potential epigenetic mechanism behind the change in function. During the study, she identified 16 genes that were differentially methylated and expressed in newborn pups, she says. This group of targeted genes may hold clues to how DEHP acts transgenerationally.

In another new study, Virender Rehan, a professor of pediatrics at the Harbor–UCLA Medical Center, found that prenatal exposure to nicotine in rats starting at embryonic day 6 was associated with asthma-like symptoms among F_3 males and females. But, similarly to an earlier study extending to F_2 offspring, the effects were sex-specific, with total airway system

resistance significantly greater in males than females, due in part to tracheal constriction, which was detected only in males.[8,9] What's still unclear (and a subject of his current research), Rehan says, is whether the transgenerational effect is being carried through the male or female germ line.



When a pregnant woman is exposed to an environmental agent, the exposure extends not only to herself (F_0) and her unborn child (F_1), but also to the germ cells developing within the fetus (F_2). Animal studies have demonstrated chemical effects that extend a generation further still—to the F_3 generation, the first generation not directly exposed to the original agent. Human studies to date have shown effects only through the F_2 generation.

Joseph Tart/EHP

Bruce Blumberg, a professor of developmental and cell biology at the University of California, Irvine, recently published a mouse study showing that maternal exposure to the biocide tributyltin (TBT) induced a condition similar to nonalcoholic fatty liver disease out to the F_3 generation.[15] Like other transgenerational toxicants, TBT is an endocrine disruptor that appears to be an obesogen, or a chemical that promotes obesity partly by promoting the growth of fat cells.[16] Blumberg's study used doses as much as 50-fold lower than the no observed adverse effect level (NOAEL) for TBT.

According to Blumberg, the findings also support an evolving concept in reproductive biology—the "developmental origins of health and disease" hypothesis, which holds that low-dose chemical exposures or maternal dietary changes experienced *in utero* can induce permanent physical changes in adult animals.[17] "These effects are permanent in that they remain even when you take away the exposure," he says. "Now we're finding that the effects can also last through subsequent generations."

Other researchers have found evidence that transgenerational effects can impact mating behaviors, with implications for the evolution of populations. In one example, David Crews, a professor of biology and psychology at the University of Texas at Austin, reported that female rats avoided F_3 males with an ancestral exposure to vinclozolin. The study specifically found that all females tested preferred control males (who had no ancestral vinclozolin exposure) whereas males from both the control and ancestrally treated groups exhibited no particular preference for female type.[18] "Where the rubber meets the road in evolution is sex," Crews says. "It's all about who mates and reproduces with who."

The Case for Multigenerational Effects in Humans

The evidence for environmentally induced multigenerational effects in humans began to emerge years ago from an isolated community in Northern Sweden called Överkalix Parish. Led in part by Marcus Pembrey, a clinical geneticist at the University College London Institute of Child Health, researchers investigated whether an abundance of food in childhood had any influence on the risk of heart disease and diabetes among a child's future descendants. In particular, the researchers studied overeating during a child's "slow-growth period," the lull before the prepubertal growth spurt.

An initial study published in 2002 suggested the answer was a conditional yes. By studying harvest statistics, grain prices, and other records, the researchers classified food availability in Överkalix for individual years of the nineteenth century as poor, moderate, or good. They then studied health outcomes among descendants born in 1890, 1905, and 1920, and found that food abundance during the grandfather's (but not grandmother's) slow-growth period was associated with an increase in diabetes mortality.[12]

In a follow-up study of the same Överkalix individuals, Pembrey and colleagues found further evidence of sex-specific multigenerational effects: Male descendants had a statistically increased relative risk of mortality if the paternal grandfather had a good food supply during his slow-growth period, while females had statistically higher relative risks if the paternal grandmother had good food availability during *her* slow-growth period.[12]

Other data come from the grandchildren of women who were pregnant in the Western Netherlands in the winter of 1944–1945, when nutritional intake dropped to as little as 400 calories per day as a result of food import restrictions by the occupying German army. In 2008 researchers led by Roseboom reported that the children of women who were exposed to famine *in utero* tended to be fatter at birth and more prone to health problems in adulthood than the children of women born before or after the famine.[19] In earlier studies, Roseboom and colleagues had reported that adult F_1 populations exposed to famine conditions *in utero* had higher rates of cardiovascular disease,[20,21] diabetes,[22,23] obesity,[24] and breast cancer.[25]

The F_1 mothers in the 2008 study completed questionnaires about the birth conditions and current health status of their grown children. The questionnaire grouped health outcomes into four categories: congenital, cardiovascular and metabolic, psychiatric, and other. The only statistically significant association between ancestral famine exposure and poor health outcomes was with the "other" category, which included accidents and acquired neurological, autoimmune, infectious, respiratory, neoplastic, and dermatological conditions. In their conclusions, Roseboom and colleagues state that the findings "constitute the first direct evidence in humans that the detrimental effects of poor maternal nutrition during gestation on health in later life pass down to subsequent generations."[19]

Roseboom calls the findings "a first but weak" indication of multigenerational effects on health after prenatal famine exposure. "It was weak because we approached the F_1 and not the F_2 directly," she explains. "But in a next study we contacted the F_2 directly, and we found they were more adipose not only at birth but also currently while in their forties, and therefore we expect that they might have increased cardiovascular disease rates later on in their lives."

Epigenetics is grounded in the work of Conrad Waddington in the 1940s, who coined the term and used it to describe non-Mendelian phenomena influenced by the environment. Many years earlier, French biologist Jean-Baptiste Lamarck had postulated that an organism can pass on traits acquired during its own lifetime.

Another key line of human evidence in the field comes from multigenerational studies of DES.[2] Those data came from a pair of National Cancer Institute studies: the DES Follow-Up Study, which tracks health outcomes among women who were exposed to DES and their prenatally exposed children, and the DES Third Generation Cohort Study, which tracks the male and female grandchildren of the originally exposed women.

According to Linda Titus, a professor in community and family medicine and pediatrics at the Geisel School of Medicine at Dartmouth, grandsons of DES-exposed women had a modestly higher risk of any birth defect, mostly urogenital defects, although the findings weren't statistically significant. Granddaughters, meanwhile, had a higher frequency of hip dysplasia, irregular periods, older age at menarche, and potentially an increased risk of infertility. There was also a higher risk of ovarian cancer among granddaughters of exposed women, but since that finding is based on just three cases, she says, it must be considered preliminary.[26]

Lamarck's theories, published 50 years before Darwin's *On the Origin of Species*, were accepted by Darwin and others until the rise in the early 1900s of Mendelian genetics, which holds that inherited traits come solely through genes.

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Epigenetic Evidence in Humans Still Emerging

The human data on epigenetics is generally limited to F_1 populations and comes mainly from studies on the Dutch famine.[5,10,11,12] According to Roseboom, the first study to link undernutrition during gestation to altered epigenetic status was published by Bastian T. Heijmans, an associate professor of genetics at Leiden University Medical Center.[5] In that study, Heijmans and colleagues reported that F_1 generations exposed to Dutch famine conditions *in utero* had hypomethylation of the *IGF2* gene six decades later, compared with same-sex siblings not exposed to famine (they noted that other stressors such as cold and emotional stress could have contributed to the observed hypomethylation).

According to Roseboom, this finding suggests prenatal famine could lead to changes in gene expression via changes in methylation. But Heijmans' research team was not able to statistically associate hypomethylated *IGF2* with any specific health outcomes. And Roseboom points out that "whether these changes in methylation actually result in changes in gene expression and ultimately changes in, for instance, cardiovascular risk factors remains to be investigated."

Roseboom's team followed up last year with a study investigating four additional genes that have been shown in animals to be persistently altered by maternal dietary restrictions. But the study failed to demonstrate any consistent links between famine exposure and methylation status, possibly because of confounding from lifestyle choices and diet later in life.[12] Roseboom's team is currently analyzing methylation levels on DNA obtained from the F_0, F_1, and F_2 generations affected by the Dutch famine; these data have not yet been submitted for publication.

Titus says that conclusive evidence of transgenerational epigenetic mechanisms in humans will depend on findings in F_3 generations. "Even if new studies confirm outcomes in DES-exposed grandchildren, we can't be sure if they are due to epigenetic changes," she says. "A true assessment of heritable epigenetic changes requires studies of great-grandchildren, which will be the first generation without DES exposure."

Blumberg emphasizes that just because the data haven't yet materialized doesn't mean that environmentally induced, transgenerational epigenetic changes in humans don't occur. "We see transgenerational epigenetic changes in animals, and what we believe is that the animal data predict human responses," he says. "Moreover, it's possible that you won't see epigenetic changes from looking at genes—you might see it, instead, in noncoding regions in DNA."

The growing evidence that environmental exposures might induce a myriad of effects that persist transgenerationally leaves open questions about where human evolution is headed, Crews asserts. "It's a new window on the 'nature versus nurture' debate," he says. "We're all combinations of what we inherit and what we're exposed to in our own lives. And right now you can't find a human or an animal on the planet without a body burden of endocrine-disrupting chemicals."

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Tags: Birth Defects, Bisphenol A (BPA), Diet and Nutrition, Dioxins and Furans, Endocrine Disruptors, Epigenetics, Genetic Research, Molecular Biology, October 2013, Pesticides, Phthalates, Reproductive Health

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Agrichemicals in surface water and birth defects in the United States

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Abstract

Go to:

Objectives: To investigate if live births conceived in months when surface water agrichemicals are highest are at greater risk for birth defects.

Methods: Monthly concentrations during 1996–2002 of nitrates, atrazine and other pesticides were calculated using United States Geological Survey's National Water Quality Assessment data. Monthly United States birth defect rates were calculated for live births from 1996 to 2002 using United States Centers for Disease Control and Prevention natality data sets. Birth defect rates by month of last menstrual period (LMP) were then compared to pesticide/nitrate means using logistical regression models.

Results: Mean concentrations of agrichemicals were highest in April–July. Total birth defects, and eleven of 22 birth defect subcategories, were more likely to occur in live births with LMPs between April and July. A significant association was found between the season of elevated agrichemicals and birth defects.

Conclusion: Elevated concentrations of agrichemicals in surface water in April–July coincided with higher risk of birth defects in live births with LMPs April–July. While a causal link between agrichemicals and birth defects cannot be proven from this study an association might provide clues to common factors shared by both variables.

Keywords: Atrazine, Birth defects, Nitrates, Pesticides

INTRODUCTION

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The leading cause of infant mortality in the United States is birth defects (1), accounting for 20.1% of all infant deaths. There is a growing body of evidence that agrichemical exposures may contribute to birth defects (2–8).

Large-scale longitudinal human studies evaluating pesticide/nitrate exposure at the time of conception have not yet been performed in the United States. Concentrations of nitrates and pesticides in stream water may be an indication of human exposure levels. The United States Geological Survey's (USGS) National Water-Quality Assessment (NAWQA) study provides the most comprehensive national-scale analysis of pesticide occurrence and concentrations in streams and ground water. In the NAWQA study, pesticide concentrations were measured in water samples from 186 stream sites representing 51 hydrological systems from 1991 to 2002. The

NAWQA study units account for 70% of total water use and 50% of the United States drinking water. Pesticides were found to be present in most stream water samples and over half of the ground water samples. Seasonal patterns of pesticide concentrations were found with the highest monthly concentrations in May and June (8). The study also found that 90% of pesticide exposure is to mixtures versus individual pesticides.

The USGS indicated a strong relationship between pesticide occurrence in water samples and their use each year. Studies of pesticide occurrence in humans also correlated with pesticide applications and peaked in the spring months (9–11).

The present study relies on the general findings by USGS, the Environmental Protection Agency (EPA) and other agencies indicating that seasonal variations in nitrates, atrazine and other pesticides may serve as markers for annual agricultural and urban pest-control activities. In the present investigation we sought to answer a qualitative question; are annual peaks in pesticides and nitrates (typically from April to July) correlated with greater risk to pregnancies conceived in those months? If no increase in birth defects were found in April–July conceptions it might be inferred that the contaminant peaks pose little threat to human reproductive success.

METHODS

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Surface water nitrates, atrazine and all other measured pesticide concentrations (agrichemicals) were obtained monthly for each year between 1996 and 2002 from the USGS NAWQA database. Monthly pregnancy and birth outcome data were obtained from the Centers for Disease Control (CDC) natality database for the same years 1996–2002. Year of delivery, month of last menstrual period (LMP), presence of any birth defect and category of birth defect were recorded for each live birth. Maternal risk factors and demographics including alcohol use, tobacco use, diabetes, age, race and metropolitan or non-metropolitan residence were also recorded. Mother's month of LMP was used as a proxy for the time of conception and all birth defect rates were calculated based on cases per 100 000 live births for each LMP month. Stillbirths and abortion data were not used.

Measures, predictors and factors

Primary measures of interest are (i) dichotomous variables of total birth defects and individual birth defects and (ii) numerical variables of concentrations of agrichemicals including atrazine, nitrate and other pesticides. The major factor of interest is the monthly or seasonal factor, that is the months April–July versus other months. Other predictors/factors include maternal risk factors, maternal demographics and year of birth.

Statistical methods

We performed three major analyses in this study. First, dichotomous variables such as total and individual birth defects were assessed for their associations with the seasonal factor (a two-level factor of 'peak' season in months April–July and 'off-peak' season of other months) in a multivariate logistic regression model adjusting for other covariates such as maternal risk factors, maternal demographics and year. Second, agrichemicals were modelled with the seasonal factor using multivariate regression models, adjusting for years. Agrichemicals were log-transformed before performing multivariate regression analyses since their distributions were right skewed. Third, relationships between birth defects and agrichemicals were assessed using multiple logistic regression models, adjusting for maternal risk factors, maternal demographics and years. Both simple and multiple models were considered in this approach. The simple model used only one agrichemical as the major predictor of interest while the multiple models used all three agrichemicals as the predictor. All statistical analyses were performed using statistical software package SAS version 9.2 (Gary, NC). p-value <0.05 was considered statistically significant.

RESULTS

Go to:

Baseline characteristics

A total of 30.11 million births were studied between 1996 and 2002. Table 1 shows women between ages 20 and 35 accounted for over 65% of total births, non-Hispanic whites over 59% and residents in rural areas less than 18%. One percent reported using alcohol during pregnancy, less than 13% using tobacco and less than 3% reported gestational diabetes.

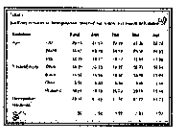

Table 1
Summary of maternal demographics, maternal risk factors and overall birth defect rates by month of LMP (conception)

Birth defects

Our study included 22 birth defect categories with the overall birth defect rate defined as any one birth defect. Table 1 and Figure 1 show the mean birth defect rates for each maternal LMP month. Birth defect rates were higher when mother's LMP was April–July. Table 2 shows that birth defect rates for April–July LMPs were significantly higher than birth defect rates for other LMP months (1621/100 000 vs. 1573/100 000 live births $p < 0.01$). Birth defects were positively associated to the maternal risk factors. Higher birth defects were found among mothers who had alcohol, smoking or diabetes. Nevertheless, mothers who didn't report having alcohol, tobacco or diabetes still had higher overall birth defect rates with LMPs in April–July than in other months. Figure 2D shows that birth defect rates decreased over the years of the study period. When individual birth defects were considered, spina bifida, circulatory/respiratory anomalies, tracheo-esophogeal defects, gastrointestinal defects, urogenital defects, cleft lip, adactyly, clubfoot, musculoskeletal anomalies, Down's syndrome and other birth defects were found to be significantly higher in April–July than in other months of the year (Table 3).

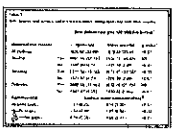

Table 2
Birth defects and surface water concentrations during April–July and other months

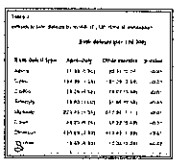

Table 3
Individual birth defects by month of LMP (time of conception)

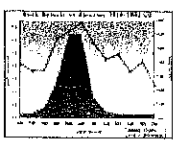

Figure 1
The United States birth defect rates by month of LMP versus atrazine concentrations.

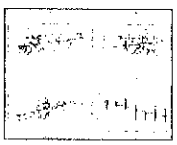

Figure 2
Plots of log odds of overall birth defect versus surface water concentrations (in log value) and year. Points in each plot represent all monthly observations in years 1996–2002. Solid lines and slopes were estimated from univariate logistic regression ...

Surface water concentrations

Table 2 shows atrazine, nitrate and other pesticides are the highest in April–July. The geometric means (standard errors) of atrazine, nitrate and other pesticides in April–June were 1.31 (0.20) μg/L, 1.94 (0.10)

mg/L, and 0.14 (0.05) μg/L respectively; and were 8.2, 1.2 and 2.8 folds of those in other months (all p-values <0.05).

Association between birth defects and surface water concentrations

Figure 2A–C) demonstrates that log odds of Birth Defects Are Positively Correlated With Atrazine, nitrate and other pesticide concentration levels in the simple models. Similar results were also found in the multiple analysis when all three geometrical predictors were included in the logistical regression model (p-values <0.05 for all three predictors). Table 4 demonstrates the effect of atrazine, nitrate and other pesticides. In general, individual birth defects rarely reached significance with individual contaminants. Only 'other congenital anomalies' reached significance in simple and multiple models with all three contaminant classes. In simple regression models, however, atrazine exposure increased the odds of 9 of 11 birth defects found to be associated with LMPs in April–July. This table contrasts with Table 4 in which all contaminants were significantly associated with any birth defects combined.

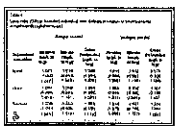

Table 4
Odds ratio (OR) of selected individual birth defects in relation to environmental contaminants (agrichemicals)

DISCUSSION

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This report has shown that during the period from 1996 to 2002 women in the United States with LMPs in April–July (i.e. the time of conception) were significantly more likely to have a live birth with a birth defect than in other months. The report further demonstrates, using NAWQA surface water samples that concentrations of atrazine, nitrates and other pesticides also were higher in the months of April–July. The correlation between birth defects, pesticides and nitrates was statistically significant.

Pesticides and nitrates, separately and in combination, have been linked to embryo toxicity and to untoward outcomes of pregnancy (12,13). Women's pesticide exposures through household gardening, professional application or living in close proximity to agricultural crops were associated with increased risks of offspring having neural tube defects and limb anomalies (14). Garry et al. found that in western Minnesota the rate of specific birth defects was elevated in pesticide applicators as well as the general population of western Minnesotans and that this risk was most pronounced for infants conceived in the spring (15). Specific birth defect categories showing significant increased risk in Garry's study were circulatory/respiratory, urogenital and musculoskeletal/integumental which are similar to the categories found in our study. Schreinemachers et al. found that infants in four wheat-producing states conceived in April–June, the time of herbicide application, were more likely to have circulatory/respiratory (excluding heart) malformations compared with births conceived during other months. She also found that counties with high wheat acreage had higher rates of heart malformations, musculoskeletal/integumental anomalies and infant death from congenital anomalies in males (16).

In Missouri men, high urine levels of atrazine, alachlor and diazinon were associated with abnormal sperm (9). The same study found that spring or summer samples were more likely to be abnormal than winter samples and that exposed men were frequently exposed to more than one pesticide causing many of the pesticide metabolites to be correlated. Thus, paternal as well as maternal exposures to pesticides might potentiate birth defects.

A causal link between birth defects and environmental nitrates/pesticides is plausible but not proven from this present ecological study. Nevertheless a statistically significant increased risk was found for any birth defect and for spina bifida, circulatory, tracheal, gastrointestinal, urogenital, musculoskeletal anomalies, cleft lip, adactyly, clubfoot and Down's syndrome in women with LMPs between April and July in the United Sates (Table 3). This period of increased risk is an important reproductive demographic.

Nitrates and pesticides occur as mixtures in most water samples (17). Recent observations in frogs, rats and other animals have demonstrated that individual chemicals at environmentally relevant concentrations may show little or no toxicity but when added together the effects are significantly more toxic or disruptive of vital endocrine functions (12,18,19). It is likely that other contaminants not specifically measured by the NAWQA study could also peak in April–July including air pollutants (20). Thus, the period of increased risk might not be associated solely with pesticides and nitrates.

This study has many limitations. Vital records have limited reliability and validity and should be used with caution (21). As of May 2001, 13 states plus the District of Columbia had only passive birth defects surveillance programmes (22).

Using NAWQA water data as a proxy for human exposure have significant limitations as well. Drinking water sources vary from surface to ground water and varied mixtures at different times of the year are common. Mean levels of nitrates and pesticides in NAWQA test sites are significantly higher than drinking water means would be in most locations. Nevertheless, in an EPA drinking water data sample from the same time period, peak frequency of pesticide detections were found in June, and correlated qualitatively with NAWQA surface water data. Atrazine was found in 57.9% of drinking water samples in Maryland (23) and 87% of drinking water samples in a sample of 12 Corn Belt states (17). A Canadian study of the northern Great Plains reported pesticides in numerous drinking water reservoirs, and depending upon location despite water treatment, 3 to 15 herbicides remained in drinking water (24).

Several studies in children and pregnant women using urine, amnionic fluid and meconium have demonstrated that from 89% to 100% of fetuses in the United States are exposed to pesticide *in utero* and most are exposed to mixtures of several pesticides (25). The importance of interactions between genetic susceptibility and *in utero* pesticide exposure has also been reported (26).

The National Health and Nutrition Examination Survey (NHANES) found that 95% of the United States population has measurable pesticide metabolites in urine samples (27). Although the atrazine mercapturate (AM) metabolite of atrazine was found in <5% of NHANES participants, Barr et al. have recently found that population-based atrazine exposures have been significantly underestimated for samples collected in the 1990's (28). The National Human Exposure Assessment Study in Maryland (NHEXAS-MD) demonstrated that over 80% of sampled individuals had at least one of three pesticide metabolites in their urine. The study found atrazine peaks occur in late summer and fall in the Baltimore area whereas the Midwest peaks occur in May and June (29).

CONCLUSIONS

Go to:

Birth defect rates in the Unites States were found to be highest for women conceiving in the spring and summer (maternal LMPs in April–July). This increase was significant for 11 of the 22 categories of birth defects reported in the CDC natality database from 1996 to 2002. A significant association was found between the months of increased risk of a birth defect (April–July) and increased levels of nitrates, atrazine and other pesticides in surface water. Critical time periods before and after conception may link seasonal peaks in environmental contaminants to certain birth defects.

Acknowledgments

Go to:

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Glossary

Go to:

Abbreviations

LMP first day of last menstrual period
NAWQA National Water Quality Assessment Programme
USGS The United States Geological Survey
CDC Centers for Disease Control
OR odds ratio
mg/L milligrams per litre
μg/L micrograms per litre
Anen anencephalus
Spina spina bifida/meningocele
Hydro hydrocephalus
Micro microcephalus
Nervous other central nervous system anomalies
Heart heart malformations
Circul other circulatory/respiratory anomalies
Rectal rectal atresia/stenosis
Omphalo omphalocele/gastroschisis
Gastro other gastrointestinal anomalies
Genital malformed genitalia
Renalage renal agenesis
Urogen other urogenital anomalies
Cleftlp cleft lip/palate
Adactyly polydactyly syndactyly, adactyly
Clubfoot club foot
Hernia diaphragmatic hernia
Downs Down syndrome
Chromo other chromosomal anomalies
Musco musculoskeletal
Othercon other congenital anomalies
Tracheo tracheoesophageal fistula
NHANES National Health and Nutrition Examination Survey
NHEXAS National Human Exposure Assessment Study
AM atrazine mercapturate

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Go to:

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