U.S. EPA Endocrine Disruptors Program 2006 STAR Progress Review Workshop

U.S. Environmental Protection Agency
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Draft Executive Summary

OVERVIEW

The U.S. Environmental Protection Agency (EPA) Endocrine Disruptors Program 2006 STAR Progress Review Workshop was held July 13–14, 2006, in Research Triangle Park, North Carolina. Sponsored by the National Center for Environmental Research (NCER), which is part of EPA's Office of Research and Development (ORD), the meeting brought together scientists from academia and government to discuss ongoing research examining the relationship between endocrine disrupting chemicals (EDCs) and human and environmental health. The meeting provided an opportunity for grantees in the EPA-funded Science To Achieve Results (STAR) Program to present their research and communicate with EPA staff and others conducting EDCs research. The meeting also permitted a chance to review related research being conducted in ORD laboratories. Approximately 85 individuals attended the meeting, and a list of participants is attached.

Welcome and Introductory Remarks
Susan Laessig, EPA/ORD/NCER

Dr. Susan Laessig welcomed the participants and summarized the agenda, which included an introduction by Christopher Zarba, NCER's Acting Deputy Director, and an overview of EPA's Endocrine Disruptor Research Program by Dr. Elaine Francis, the program's National Program Director. Each of the five sessions was chaired by a researcher from one of EPA's laboratories/centers and included a presentation by the chair followed by the STAR grantee presentations. Day 1 of the meeting featured a panel session in which program and regional representatives discussed EDC research in a regulatory context, highlighting the use of this research in environmental policy decisions, and sharing their perspectives on possible future research and regulatory directions.

Opening Remarks
Christopher Zarba, EPA/ORD/NCER

Dr. Christopher Zarba welcomed the participants and thanked them for attending the meeting. He commended the intent of progress reviews, such as this meeting, which provide an opportunity to learn about how the science is evolving and to be reminded of the main goal of the research underway—to help EPA protect human health and the environment. He then gave an overview of the EPA planning process. Early in the process, ORD meets with customers in the program offices and regions to help them identify their research needs. A list of priorities results after a round of ranking and strategizing. EPA then decides which of the highest ranking research will be done in-house and which will be handled by extramural investigators. The program offices and regions assist in writing the Request for Applications (RFA), which gets published on the NCER Web Site. Applications received in response to the RFA are ranked and prioritized by a panel of external peer reviewers. Only applications scored as very good or excellent are considered for funding. A relevancy review then determines which of the peer-reviewed application

selections will be the most effective in providing the required research answers. An internal Agency review and another ranking are followed by a final cut to decide which of the top application choices are granted funding. Dr. Zarba concluded by describing research by the grantees as some of the best examples of the Agency doing its job and one of the wisest uses of U.S. taxpayers' money to focus on the most critical environmental issues of the nation.

Overview of the Endocrine Disruptor Research Program
Elaine Francis, EPA/ORD/NCER

Dr. Elaine Francis welcomed the participants and provided an overview of the research in EPA's Endocrine Disruptor Research Program (EDRP). She described the scientists from the external scientific community as an excellent complement to the research being undertaken within the Agency. Unique among research organizations, the program features a multidisciplinary set of research areas for both human health and wildlife, as well as a range of research partners and approaches. EPA is focusing on EDCs because of evidence suggesting that environmental exposure to chemicals that mimic hormones cause adverse effects in wildlife and also may harm humans. This core research program is paralleled by the Endocrine Disruptor Screening Program (EDSP), which was developed in response to a Congressional mandate in the Food Quality Protection Act (FQPA) and Safe Drinking Water Act Amendments (SDWAA) of 1996. Complementing the intramural work ongoing within ORD has been research conducted through the STAR Program. As a whole, EPA's EDCs research program is addressing three key long-term goals (LTGs) as identified in the Agency's Multiyear Plan (MYP) for Endocrine Disruptors. LTG 1 seeks to provide a better understanding of the science underlying the effects, exposure, assessment, and management of EDCs. Studies addressing this LTG include the characterization of neuroendocrine influences of exposures during development, which result in altered genital and pubertal development. LTG 2 aims to determine the extent of impact of EDCs on humans, wildlife, and the environment. Research in this area is examining compounds in paper mill effluents, wastewater treatment effluents, drinking water, and concentrated animal feeding operations (CAFOs); it also seeks to extrapolate findings from the laboratory to the population level. The goal of LTG 3 is to support EPA's EDSP, including studies on *in vivo* and *in vitro* assays and establishing dose-response relationships for hazard assessment. Future steps for the EDRP include continued development of new methods and tools and applying them to environmentally relevant issues, such as pharmaceuticals, and developing a Web site.

SESSION I: EFFECTS OF EDCS ON REPRODUCTION

Session Introduction
Susan Laws, EPA/ORD/NHEERL

Dr. Susan Laws introduced the meeting's first session, which focused on epidemiological studies assessing the link between EDC exposures and reproductive outcomes. The presentations in this session explored the potential associations between EDCs, such as pesticides and organochlorines, and genital abnormalities, endometriosis, and aspects of the reproductive system.

Effects of EDCs on Reproduction: Reducing Scientific Uncertainties
Susan Laws, EPA/ORD/NHEERL

Dr. Laws provided an overview of what currently is known regarding the environmental effects of chemicals on reproduction and touched on the type of information needed to reduce some of the existing scientific uncertainties in this area. Highly specialized, the endocrine system works to regulate the processes of metabolism, digestion, reproduction, stress, and physiological homeostasis. Hormonal feedback loops function to maintain a homeostatic balance in the system, which presents a range of

targets for EDCs, such as receptor-mediated mechanisms and hormone synthesis, clearance, and transport. Several lines of evidence point to the detrimental effects of EDCs on reproduction. Laboratory-based toxicology studies have revealed findings such as malformations in the reproductive tract following *in utero* and/or lactational EDC exposure. EDC effects following exposure during adulthood have included disruption of ovulation and/or cyclicity in females and reduced testosterone and infertility in males. Adverse endocrine effects of EDCs also have been observed in wildlife, with a range of understanding of the mechanism of action (MOA). Findings supporting wildlife effects include poor reproduction in fish and birds in the Great Lakes (MOA unclear) and increases in vitellogenin production and occurrence of intersex in fish in a sewage treatment plant in England (MOA strong). Observed possible human health effects include reproductive disorders such as declining sperm counts, testicular cancer, and changes in fertility rates in males; and spontaneous abortions, endometriosis, and breast cancer in females. Overall, the evidence presented in relation to human health effects of EDCs remains weak, with MOAs unclear. To help determine whether EDCs have an effect on health, the Hill Criteria of Causation outline the minimal conditions needed to establish a causal relationship between a specific factor and a biological outcome/disease. The assessment factors used include temporality, strength of association, and biological plausibility. Several areas related to the biological plausibility of EDC action require more data to reduce scientific uncertainties; these include exposure-outcome relationships, comparative toxicology, chemical diversity, MOAs, and cumulative exposures and effects.

Persistent Organic Pollutants and Endometriosis Risk
Victoria Holt, Fred Hutchinson Cancer Research Center

Pelvic endometriosis occurs when endometriotic tissue—tissue that is normally on the inside of the endometrium and is shed during menstruation—is implanted in regions outside the uterus, sometimes on the ovary and fallopian tube, and possibly within the pelvic cavity. The tissue forms adhesions that can cause chronic pelvic pain, painful intercourse, ovarian damage, and infertility. Endometriosis occurs in about 5 to 10 percent of reproductive-aged women and involves severe menstrual pain and severe bleeding. The disease is not curable, but is treatable by gonadotropin-releasing hormone (GnRH) agonists, Danazol, and progesterone, among other substances. Surgical remedies include lesion resection/ablation, hysterectomy, and oophorectomy. Past studies have noted that markers of high estrogen levels—including body fat distribution, height, and alcohol use—increase the risk of the disease. Laboratory findings have revealed connections between persistent organic pollutants and estrogen, including estrogenic activity in estrogen-receptor binding tests and estrogenic effects on breast cancer cells. In animal studies, dioxin effects have been found in mice and primates, with no polychlorinated biphenyl (PCB) effects in mice and uncertain PCB findings in primates. Human studies also have revealed significantly increased endometriosis effect associated with dioxin, with some increased effects related to PCBs. The present study, Women's Risk of Endometriosis, involved 540,000 women in Western Washington State between 18 and 49 years of age. Information was collected via in-person interviews, dietary questionnaires, body and blood measurements, and medical/pharmacy record abstraction. Data analysis revealed a fairly consistent pattern of modestly increased risk affiliated with self-reported exposure and with the top tertile of pesticide and PCB serum levels. Although the results were age-adjusted, further modeling will be done to account for possible other noncausal explanations for the associations found.

Discussion

A participant asked whether any relationship was observed between endometriosis risk and the onset of puberty or menarche and whether the study examined age of menarche in relation to the disease. Dr. Holt replied that early menarche is a known risk factor for endometriosis but this relationship was not examined in her study. The same participant asked if the study is reexamining the distribution of the exposure data in case there are any peaks in the data, in addition to the top tertile, which might be of interest. Dr. Holt responded that her group is interested in taking this step. For some of the findings, less

than a quarter of the people were below the limit of detection, so quartiles could work in these analyses. If, however, more than a fourth of the subjects are below the limit of detection, examining quartiles would not be helpful. Dr. Holt welcomed suggestions with respect to analytic methods.

Dr. Henry Anderson asked whether the study has considered body mass index (BMI) because one of the issues for the various chlorinated compounds is that there are different metabolic and clearance rates of compounds associated with the degree of body weight. Dr. Holt responded that the study did include BMI but did not find much difference between the treatment and control groups. She explained that, historically, BMI is correlated with endometriosis, with thinner women being more likely to be diseased; however, this might be an artifact of how women come to diagnosis. Often thinner women seek infertility treatment, which is associated with higher socioeconomic status and lower BMI.

A participant asked whether the study attempted to test a combined exposure in a clean reference group, such as those who answered "no" to fungicide and herbicide exposure in the questionnaires. Dr. Holt responded that the study only assessed one exposure at a time, although exposure to different types of PCBs (antiestrogenic and estrogenic) was considered to some extent. The same participant commented that it is beneficial to read the 1965 paper by Sir Austin Bradford Hill. In the paper, Hill stated why one would not expect to see a dose response in epidemiologic studies and how this differs from animal studies. Also, he points out clearly that the material he discusses is not intended as criteria. The participant added that, over the years, it seems Hill's message has been misinterpreted.

Another participant asked whether the study controlled for characteristics other than endometriosis, such as birth control and reproductive histories, because these may have been precursory to the development of endometriosis. In other words, how was it ensured that these cases were not present in the control group? Dr. Holt responded that all of the relevant information was collected and that she does not think there would be that many cases in the control group. Subanalyses could be done that would exclude women with any precursor symptoms from the control group. The same participant commented that pharmaceuticals, such as the cholesterol-lowering drug Lipitor, can be quite potent and are inducers of steroid metabolites and enzymes that affect estrogen levels. The participant added that pharmaceuticals can change patterns of metabolism for endogenous steroids. Did the study measure any endogenous steroids in the blood samples? Dr. Holt mentioned that pharmacy records for all study subjects were available and that Lipitor was not likely used in women under the age of 50. The study did not measure endogenous steroids. In the case study of women with endometriosis 1 year later, some of the women had hysterectomies and oophorectomies and, therefore, were surgically postmenopausal. Thus, measuring hormone levels in these women would not be needed.

A question was raised about occupational history of exposure to pesticides. Because the women knew about their endometriosis, were they asked about their chemical exposures on the job or was the study creating a job exposure matrix? Dr. Holt responded that the former was done, but that the latter is a good approach to eliminate the bias from women who were diseased and were now going back over their lives trying to assess any exposure they possibly have had. Dr. Holt welcomed collaborations in creating such matrices.

Latent Effects of Gestational Exposure to Heptachlor
Dean Baker, University of California, Irvine

An occurrence of population-level *in utero* exposure to heptachlor epoxide (HE) in the early 1980s was the focus of this study. At the time, HE was used in Hawaii as a pineapple pesticide. The commercial milk supply on the island of Oahu was contaminated with HE during a 15-month period from 1981 to 1982, resulting in gestational exposure to offspring of women who drank milk during that time. Research conducted in 1989 to 1991 demonstrated significant associations between HE concentrations in both

human milk and serum of adults for those who reported cow milk consumption from 1981 to 1982. A neurobehavioral effects study from 1998 to 2002 that examined Oahu high school students born during 1981 and 1982, and who lived on Oahu, found that mothers' reported cow milk consumption during pregnancy was associated with worse neurobehavioral performance and visual perception, as well as more behavioral problems. The present study ran from 2003 to 2006 and followed young adults from Oahu who participated in the earlier neurobehavioral study, to examine HE effects on reproductive and immune functions. The primary exposure variables were birth location (Oahu-born vs. elsewhere) and mothers' reported cow milk consumption at time of pregnancy. Reproductive function indicators included serum measurements of luteinizing hormone (LH) and follicle-stimulating hormone in both sexes; estradiol in women; and testosterone in men. Urine and semen indicators also were measured. Immune function indicators included antibody titer response to immunization with tetanus and pneumococcal vaccines. To date, 456 participants have completed most of the protocol, with 87.5 percent of participants born on Oahu. Among the findings, slightly earlier puberty milestones in females and minimally later development in males were associated with mothers' reported cow milk consumption during pregnancy, but none of these associations were statistically significant. Altogether, no substantial effects of gestational HE exposure were found on reproductive or immune functions. Future work will include completion of serum and urine analyses on remaining sample specimens to finalize analysis of the immune function parameters.

Discussion

Dr. Anderson asked whether whole milk was sold during the 1980s or was it possible that some of the women were drinking low-fat milk. He added that the results might have been stronger if this detail had been sorted out. Dr. Baker responded that the study examined milk type (whole vs. skim vs. low fat), although these milk variations were not as common in Hawaii back in the early 1980s. An attempt was made to present a conservative assessment for the neurobehavioral studies by using a sensitivity analysis for milk type. If an individual had consumed a mixture of different milk types, then the glasses of milk per day would be adjusted with a multiplication factor. By effectively adjusting for the fat content in milk, the negative neurobehavioral effects became strongly significantly positive.

Dr. Jeffrey Fisher asked whether there were other possible sources of exposure in addition to milk consumption, such as via drinking water. Dr. Baker responded that heptachlor is very lipophilic, so there would be very little of this type of pesticide entering the drinking water. He added that drinking water measured at the time by the state did not contain these chemicals. There are other sources of exposure, however, including chlordane, which is metabolically related and used as a termite treatment. As expected, the team found termite treatment to be associated with chlordane serum levels. They also looked at heptachlor and several other pesticides on neighboring islands; only heptachlor was higher on Oahu than on those islands. Another potential source that was assessed included consumption of hamburgers. When cows get too old to milk, they are used for meat production. Both the children's and mothers' occupational histories also were assessed, but this information was insufficient to give meaningful pesticide exposures.

Dr. Fisher then asked whether the study is tracking body burdens. Dr. Baker responded that this is not being done because it is not a suitable biological indicator for HE exposure. Because gestational exposure occurs during a developmental period of potential susceptibility, which could cause the later development of endocrine or neurological effects, the exposure is not necessarily as high in absolute magnitude as it might be when considering effects on adult animals or adult humans.

Dioxins, Male Pubertal Development, and Testis Function
Russ Hauser, Harvard School of Public Health

Research was undertaken to investigate the effect of gestational exposure to dioxin-like compounds and nondioxin-like PCBs on the timing and tempo of pubertal development; linear growth, weight gain, and BMI; and biochemical changes in hormones that regulate growth and pubertal maturation. This study targeted a population in Chapaevsk, Russia, an industrialized city occupied heavily by military and chemical industries and with highly documented environmental levels of dioxins and PCBs. Recruitment for a prospective cohort study on adolescent boys began in 2003 and was completed in May 2005. To date, 516 boys and their mothers have been recruited. Baseline examinations of the boys included anthropometric and physiological measurements. Baseline blood and urine samples also were taken, for both mother and son, and both had to complete questionnaires on medical history, lifestyle, and dietary information. Annual visits included physical examinations, blood drawn every 2 years, and urine samples (for the son only), as well as questionnaire updates. To date, normative growth curves have been generated for height, weight, and BMI among Chapaevsk boys aged 10 to 17. The study has confirmed a wide distribution of dioxin levels among these boys and identified predictors of dioxin levels, including dietary measures, reproductive history, and residential location. Longitudinal follow-up is planned with an assembled cohort of prepubertal boys; this testing will include an annual physical examination and blood sample collection to measure serum dioxins and PCBs. This follow-up work has received 5 years of funding from the National Institute of Environmental Health Sciences. Future plans include exploring the relationship between pubertal exposure to dioxins and intermediate measures of reproductive function in the children when they reach adulthood at age 18. At that stage, testicular function will be assessed via semen evaluation and measurement of reproductive hormones.

Discussion

A participant commented that, for a number of years, there have been discussions on the feasibility of collecting sperm in urine as an indicator of puberty, an idea that has been supported in the literature. She proposed that the present study could include sperm collection via use of a funnel with detachable filter at the bottom, and that the samples could be saved in plastic bags on a monthly basis and later analyzed. Dr. Hauser responded that his group is considering the collection of sperm. They already collect urine as spot samples once per year, but multiple samples are required to detect the presence of sperm. The participant added that the collection is something the boys could do at home. Dr. Hauser agreed with this suggestion. He added that the urine also is being collected for analysis of other compounds as well, such as phthalates and bisphenol A.

Dr. Laws applauded the speakers for taking on such challenging projects, whose progress will be followed with enthusiasm. She then adjourned the session.

SESSION II: EFFECTS OF EDCS ON PUBERTY

Session Introduction
Tammy Stoker, EPA/ORD/NHEERL

Introduced by Dr. Tammy Stoker, this session featured presentations addressing associations between EDCs and male development in a human population and discussed the use of animal models to examine how EDCs alter puberty in females.

The Effects of EDCs on Pubertal Development: Current Research Efforts by EPA
Tammy Stoker, EPA/ORD/NHEERL

A time of dramatic neuroendocrine development, puberty results in reproductive maturation and requires extensive interaction between various hormones, organs, and tissues. Puberty also is a period of increased sensitivity to environmental agents and can be affected by gestational, lactational, or peripubic exposures to certain toxicants. EPA's ongoing research in this area includes in-house studies for ORD's MYPs for endocrine disruptors, human health, and safe pesticides/safe products; the EDSP; and STAR Program research. Animal models are useful for pubertal development because a delay or acceleration in puberty can be measured easily by landmarks of puberty in the rodent, including vaginal opening in the female and preputial separation in the male. Examples of mechanisms of pubertal alterations include estrogen agonists and steroidogenesis inhibitors, which advance puberty and delay puberty, respectively, in female rodent models. Also helpful is the cross-species conservation of mechanisms associated with puberty, suggesting that rodent models should be predictive of similar outcomes in humans. Evidence of altered neuroendocrine control of pubertal progression includes the action of atrazine and metabolites, which decrease GnRH, in turn resulting in decreased LH and, ultimately, delayed puberty. The neural inputs involved in GnRH secretion in the adult are possible target sites for environmental or pharmaceutical insult. The EDSP currently is developing and testing methodologies for the assessment of EDCs in several *in vivo* and *in vitro* assays. Two of the *in vivo* screens are the rodent male and female pubertal protocols, which will identify EDCs that alter pubertal development and thyroid function through the central nervous system or steroid-mediated MOA, and will quantify the effects of these chemicals. Assay endpoints being measured include body weight gain, organ weights, tissue histology, and thyroid measures. More human epidemiology studies are needed to address issues of adverse outcomes following changes in puberty. Also important is to examine whether changes in pubertal timing are linked to adverse outcomes in later life, such as increased cancer risk.

Low Dose Effects of* In Utero *Exposure to Cadmium on Puberty
Mary Beth Martin, Georgetown University

Findings have demonstrated that cadmium (Cd) has strong estrogen-like activity *in vitro* and *in vivo* and that early life exposure to environmentally relevant amounts of Cd advances the onset of puberty, increases weight gain, and changes mammary gland development in female rat offspring. This study tested the hypothesis that early life exposure to low Cd doses alters the hypothalamic-pituitary-gonadal axis, thereby causing the above-mentioned effects. In human breast cancer cells (MCF-7 cells), Cd has been found to activate estrogen receptor-alpha (ER-α) through a high-affinity interaction with the hormone binding domain (HBD). In the present study, female Sprague-Dawley rats were ovariectomized on postnatal day (PND) 28, permitted to recover for 3 weeks, and then treated with a single intraperitoneal dose of Cd at 5 µg/kg body weight. Treatments also included the ER antagonist, ICI 182 780; a combination of Cd and the ER antagonist; and estradiol alone. Uterine wet weight was examined 4 days post-treatment. Treatment with Cd produced almost a twofold increase in uterine weight, with similar hypertrophy of the endometrial lining as was obtained from treatment with estradiol. Cd also caused a significant increase in mammary gland density on day 4 post-treatment. To examine early life effects of Cd, pregnant rats were treated with 0.5 or 5 µg/kg body weight of Cd on days 12 and 17, and female offspring were cross-fostered. Both treatments showed an earlier opening of the vagina relative to the control vehicle. The higher dose treatment gave a significant increase in alveolar buds at day 35 relative to the vehicle. Challenging the animals with 10 mg/kg body weight of dimethylbenzanthracene (DMBA) promoted mammary tumor development. Recently, the team has discovered a new class of endocrine disruptors referred to as metalloestrogens, which occur in two classes—bivalent cations such as Cd and mercury; and metal/metalloid anions, including arsenite and selenite. The researchers have found that some, but not all, bivalent metals and anions activate ER-α via formation of a high-affinity complex

with the HBD. Ongoing work includes investigation of the critical time of exposure to Cd on development.

Discussion

A participant asked for clarification on whether Cd interacts with the androgen receptor. Dr. Martin replied that Cd does activate the androgen receptor; however, the amino acid binding positions are not yet known. The same participant asked how Cd compares to testosterone or dihydrotestosterone in interaction with the androgen receptor. Dr. Martin responded that the K_d values are the same. The participant then asked whether the dose required to block the effects of Cd was the same or higher than that needed to block estradiol. Dr. Martin replied that comparison material was not available in the literature, so estimation was made on the dose required to block the effect of Cd with the antiestrogen. The ICI dose that was used completely blocked the Cd effect, but the estradiol effect was never fully blocked.

Dr. Martin was asked whether the dose of Cd administered to the rats was within the environmental range. She replied that the chosen dose was based on the Provisional Tolerable Weekly Intake of 7 μg/kg body weight set by the World Health Organization, with the aim of trying to mimic what was generally found in dietary levels. The participant noted that it might be useful to learn what the current U.S. population Cd intake is from the National Health and Nutrition Examination Survey (NHANES). Dr. Martin replied that the figure used was slightly higher than that in the NHANES data.

Another participant reminded everyone of Dr. Martin's comment on having assessed arsenic (As) as well as Cd and asked her to share information on that work. Dr. Martin replied that the incidence of the findings with As is about the same with Cd, but the latency of effects decreased dramatically.

Dr. Anderson commented that many of the heavy metals that are bivalent bind very aggressively to sulfhydryl groups. Because most of the enzymes have those groups, and if many of these enzymes are placed into the *in vitro* systems, the observed MOA could be the result of blockage of some of the enzymes involved in the estrogen activity cycle. He asked how certain it is that the results reflect site blocking versus an increase in the amount of an estrogen. Dr. Martin responded that her group is spending a considerable amount of time trying to elucidate the MOA. The team has examined the crystal structure of the HBD to determine what conformational changes occur when the hormone binds the receptor. The process appears to involve at least three conformational changes in the structure.

Another participant asked about the strength of the data that Cd stimulates the same genes as estradiol. Dr. Martin replied that her team has not yet found a gene, but they have assessed four or five of them.

Study of Phthalates in Pregnant Women and Children
Shanna Swan, University of Missouri

The toxicity from prenatal exposure to phthalates results in a cluster of abnormalities collectively termed as the phthalate syndrome. Some of the effects of phthalates include a lowering of fetal testosterone and a shortened anogenital distance (AGD). In rodents, AGD is approximately twice as long in males as in females. The present study is the first to examine AGD and related endpoints in relation to *in utero* phthalate exposure in humans. Specifically, the work sought to determine whether prenatal phthalate exposure alters human male development. Mothers recruited for the study at a prenatal visit agreed to a follow-up study and provided a prenatal urine sample. The study population was a group of 166 boys, 106 of whom had prenatal phthalate exposure. Physical examination of the boys involved measurements of weight, height, and genital features, including AGD. To assess phthalate exposure, samples were collected at mid-pregnancy, and a concentration of nine phthalate metabolites was measured. The most significant results from a mixed model regression analysis were for two oxidative metabolites of di-2-

ethylhexyl phthalate (DEHP)—mono-2-ethyl-5-oxohexyl phthalate (MEOHP) and mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP)—giving a strong and inverse relationship with AGD. Other results included a significant correlation of AGD with degree of testicular size, penile volume, and scrotal size. The urinary phthalate concentrations associated with shorter AGD were consistent with those that have been measured in one-fourth of the female population in the United States. The study also found that, in relation to the EPA reference doses for phthalates, the median intake estimates associated with reduced AGD are roughly two orders of magnitude lower. Future studies are needed to determine clinical correlates of the phthalate syndrome in humans. A study is set to begin in Denmark this September, which will measure the AGD of young men coming for a military physical examination, and also will include taking sperm samples and measuring penile volume.

Discussion

Dr. Robert Zoeller commented that it is encouraging to see results that provide an overt measure of an effect on androgen signaling. He added that endocrine signaling also can occur elsewhere, such as in the developing brain, and asked if the team has considered whether cognitive or spatial abilities also might be different between girls and boys. Dr. Swan explained that her group is planning a pilot study on play behavior, which is altered in rodents that are exposed prenatally to vinclozolin and flutamide. To garner information, the team is sending questionnaires to the mothers of the children to ask standardized questions on their children's choice of games and toys.

Dr. Anderson asked whether the study has looked at other parameters, such as birth weight, because developmental parameters also may play a role in the findings. Dr. Swan responded that her team, along with a student of Dr. Russ Hauser, is examining preterm birth; other anthropometric parameters in the study population have not yet been examined. Dr. Anderson suggested that lifestyle behaviors of the mother, such as the use of medications and cosmetics, could also be considered. Dr. Swan replied that her team has not seen any effect with any of the socioeconomic factors they have examined.

Dr. Stoker concluded the session by stating that it will be interesting to learn of follow-up on the research presented today.

SESSION III: BIOMARKERS OF EXPOSURE TO EDCS

Session Introduction
Jim Lazorchak, EPA/ORD/NERL

Dr. Lazorchak introduced this session as one that will discuss research at the system level, aiming to protect ecosystems and communities. His presentation gave highlights of various ongoing system-level work, highlighting projects addressing ecological effects, ecotoxicogenomics, and bioinformatics; wastewater and chemistry fate; and drinking water/chemistry fate. The two presentations that followed were focused more intently on specific studies. Dr. Lazorchak commented that fish are exposed to mixtures, which makes it difficult to attribute causality of effects to one particular EDC or other chemical. Dealing with mixtures is, therefore, one area of science in which EDC research is moving.

U.S. EPA's Research on the Ecological Exposure and Effects of Endocrine Disruption Chemicals and Pharmaceuticals
Jim Lazorchak, EPA/ORD/NERL

Ecological effects research at EPA involves studies that are focused on developing and testing assays for EDCs. One project is examining a short-term reproduction assay for fathead minnow that detects a chemical's ability to interfere with the sex steroid axis. Another project is assessing a short-term

metamorphosis assay for *Xenopus laevis* to detect a chemical's ability to interfere with the thyroid hormone (TH) axis. Mammalian *in vitro* assays under investigation include a study making use of transgenic cell lines cultured with effluent and then tested for luciferase activity. In the area of ecotoxicogenomics and bioinformatics is a study examining the effects of whole-lake additions of ethynylestradiol on vitellogenin gene expression and reproductive success of fathead minnow. Another study is linking long-term, ecologically relevant whole-organism endpoints in fathead minnow, to short-term, molecular endpoints in the fish, using zebrafish and molecular tools on this species as a surrogate. Wastewater and chemistry projects at EPA include a study examining exposure of fathead minnow to effluents from wastewater treatment plants (WWTPs) in nine EPA regions. Monitoring human-use pharmaceuticals and drug abuse in WWTPs and source waters also is a focus for EPA. Among the drinking water/chemistry research projects in progress is a study examining the use of chemicals as indicators of the impacts on water by human fecal material. Underway as well is an investigation of the persistence of wastewater compounds, such as pharmaceuticals, through drinking water treatment. Several other ecological exposure collaborations have been initiated with EPA regions, other federal agencies, and academia using "omic" markers.

Discussion

Dr. Duncan Ferguson commented that there are some distinct differences between the use of pharmaceuticals in food animals in the United States versus what might be happening in Europe. Dr. Lazorchak stated that from his visit to the Society of Environmental Toxicology and Chemistry (SETAC) Europe 16th Annual Meeting, held May 7–11 in The Hague, The Netherlands, he learned that the United States uses much more veterinary chemicals in its animal feed lots than in Europe. In his own work, Dr. Lazorchak is concerned with whether a pharmaceutical, or in this case, a particular antibiotic, can reduce a species or group of species significantly and be measured, and the result would be based on veterinary or antibiotic exposure. For instance, cows are placed on trenbolone for 100 to 300 days, and once they are taken off the dairy cycle, they are removed from the steroid. By the time the cattle go to slaughter, the trenbolone might not be detectable.

Dr. Swan stated that she would be curious to hear from Dr. Susan Glassmeyer on what is removed by bleaching treatment of drinking water. Dr. Glassmeyer responded that sampling for that study will begin next year. Most studies in this area have been done in Europe, where they use ozone for disinfection; in the United States, chlorine is used for this purpose. For this reason, studies are underway to improve understanding of what happens during the chlorination process. It is possible that through the process, byproducts are produced in the environment that might have greater toxicity than the drugs being developed.

Dr. Bryan Boulanger pointed out that the level of these chemicals in drinking water is rather low from a human dose point of view; it is in human discharge that the levels are much higher.

Dr. Anderson commented that much focus has centered on disposing old drugs. Rather than flushing medications down the toilet, collection programs are in place for materials such as hazardous wastes. He asked if there is an estimate of the proportion of drugs that might still be going down the drain. Dr. Lazorchak responded that he does not have the information at hand, but that estimates exist. The bioinformatics project of Dr. Mitch Kostich has developed a process to estimate how much of the pharmaceuticals end up in sewage.

Dr. Francis pointed out that there is an interagency working group on pharmaceuticals that includes EPA and other federal agencies. One of the projects in development is a research strategy on how to deal with pharmaceuticals in the environment; this will serve as a blueprint for EPA to identify how to deal with certain pharmaceuticals.

Developing Rapid Assessment Tools To Evaluate the Biological Effects of Complex and Biologically Active Mixtures
Heiko Schoenfuss, Saint Cloud State University

The primary goal of this research project is to assess the hypothesis that mixtures of estrogenic compounds will have harmful effects on aquatic vertebrate fitness that can be assessed rapidly by measuring changes in the neuroendocrine system of fishes. Ultimately, the goal is to develop rapid and biologically relevant neurochemical assessment tools to correlate with reproductive fitness. Phase 1 surveyed the concentrations of alkylphenol polyethoxylates, estrogens, and pharmaceuticals from the effluent of two WWTPs—a plant in Boulder Creek, Colorado, and the Metropolitan Wastewater Treatment Plant in St. Paul, Minnesota—both of which have a history of estrogenicity. Natural estrogens were found to be at or below the detection limit for most samples and nonylphenol ethoxylates were consistently measured in the hundreds of ng/L. Preliminary work in Phase 2 is addressing food quality by examining the effect of exposing diatoms to a chemical or mixture. Diatoms are one of the primary food sources for all freshwater fish larvae. At the lowest treatment concentration of 2 μg/L of nonylphenol, the chlorophyll A to lipid ratio was more than doubled relative to the control, revealing a reduction in nutritional value of the diatom. Another component of the study is assessing the effects of exposing fathead minnow larvae in a flow-through system for 28 days to compounds individually and in mixtures, using the highest no-effect concentration for each compound for the mixtures. The fish are given the chance to spawn and then, at 35 days postexposure, measurements will be taken on vitellogenin, sexual characteristics, reproductive and locomotor ability, and neuroendocrine endpoints. The neuroendocrine endpoints currently are being developed and will include characterizing sexual dimorphism in the neuroanatomical distribution of enzymes and receptors responsible for expression of steroid-dependent behaviors. Phase 3 will include field evaluation of the neuroendocrine endpoints in exposure experiments at both WWTP field sites.

Systems Approach To Assessing Cumulative Exposure to EDCs
Gerald LeBlanc, North Carolina State University

Existing measures to assess the influence of cumulative exposure to EDCs by conventional analytical chemistry or reporter-gene approaches are insufficient because of the complex nature of EDC mixtures, which can elicit toxicity via a range of mechanisms. To address this limitation, the current study is examining cumulative exposure to EDCs by taking a systems approach that assesses multiple endocrine signaling circuits simultaneously. This approach will incorporate toxicokinetic and toxicodynamic actions and also will provide information on MOAs and interactivity. The main objective is to develop a gene-expression based, whole-organism approach to evaluating cumulative exposure to EDCs. Applying the water flea *Daphnia magna* as the exposure sensor, the study has used targeted reverse transcription–polymerase chain reaction (RT-PCR) approaches to identify candidate genes that function in endocrine signaling or respond to endocrine signals. To date, 16 genes have been identified, including NR2B (RXR), a 400 amino acid protein for which the cDNA has been cloned in entirety and sequenced. The next goal of the project is to evaluate the nature and degree of responsiveness of the genes to endocrine-active chemicals with known MOAs. Thus far, completed laboratory exposure experiments have included assays with EcR agonists, ecdysteroid synthesis inhibitors, and terpene mimics. The final aim of this research is to test the ability of the sensor to detect and assess exposure associated with EDC mixtures. This portion of the study will involve the Integrated Addition and Interaction Model, an algorithm that factors in the different MOAs of chemicals in a mixture and the fact that these chemicals might be interacting. Overall, it is hoped that the research will result in the establishment of a paradigm for application to other sensor species.

Discussion

A participant commented that many TH disruptors act through nuclear receptors such as PXR, which is a partner for RXR. Is there a homolog for PXR in the data and could it play a role in endocrine disruption? Dr. LeBlanc responded that the closest to a homolog to PXR in arthropods is probably HR96, which is one of the genes that his group is assessing. HR96 has the same origin for structural similarity to PXR; however, whether the functions are the same is not known. The function of HR96 is not well understood; it is thought to be a sensor, helping to detect unknown factors that help regulate the animal through its life cycle.

Dr. Zoeller commented that compounds can bind to a receptor and regulate genes that are different from the ones they would normally regulate. When a molecular technique is focused on a particular group of genes, and a compound alters the structure of the gene receptor enough such that the expression of a different set of genes is affected, is it a concern that these secondary influences might occur and that these effects might not be detected? Dr. LeBlanc responded that this point warrants concern, adding that he is not certain whether others have demonstrated that a foreign chemical could bind a receptor and alter it in a way that it could now bind to response factors to which it would not normally bind. Dr. Zoeller added that a possible scenario might result from pharmaceuticals causing estrogen receptors to bind to different proteins and act as co-factors. This could result in protein interactions and modified gene expression. Dr. LeBlanc responded that the system his group is developing might ultimately be able to tease apart and follow these types of secondary interactions resulting from exposures to EDCs.

PANEL DISCUSSION OF SESSION I

Moderators: Susan Laessig, EPA/ORD/NCER, and Barbara Glenn, EPA/ORD/NCER
Panel Members: Dana Heriegel, EPA/Region 3 Water Protection Division; Diana Eignor, EPA/Office of Water; Louis Scarano, EPA/Office of Pesticide Programs; Gary Timm, EPA/Endocrine Disruptor Screening Program

Gary Timm

Mr. Gary Timm noted that the main task of EDSP has been to translate recommendations of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) into workable policy. The development and validation of assays has been a substantial part of the program. He explained that ORD has been pivotal in developing the various assays and also has helped to standardize the assays of some of the older protocols, such as the Hershberger assay. Currently, validation studies are being completed, such as for the pubertal studies, and the hope is to bring that work to peer review early next year, with the first screening battery slated for December 2007. The goal is to assay 50 to 100 chemicals to see how the program works and to tweak the assays on the basis of real-world data. Once the assays are validated, the regulated industry—the manufacturers and the registrants of pesticides—will do the testing.

Diana Eignor

Ms. Diana Eignor explained that part of the responsibility of the Human and Ecological Criteria Division (HECD), Ecological and Health Processes Branch is to develop guidance criteria covering a range of materials from contaminants to nutrients to sediments. Once criteria are developed, work is undertaken with HECD's sister division, the Standards and Health Protection Division, which helps regional offices and states incorporate water quality criteria into their water quality standards. Ms. Eignor emphasized that it is important for researchers to publish their results because peer-reviewed information is referred to by the branch when it conducts risk assessments. Of particular interest at HECD is emerging contaminants and low doses, as well as looking at historical problems such as DDT. She noted that the research EPA is

conducing for LTGs 1 and 2 brings topics to the forefront and they then become a priority for the program offices. There is a need to focus on the prevalence of the chemicals and their frequency and potency. Examining mixtures also is important. Although the HECD deals with the criteria, it associates closely with the individuals who write the standards. She added that chemicals are regulated individually as well as categorized collectively by similarity in MOA.

Louis Scarano

Dr. Louis Scarano spoke broadly on the topic of registering pesticides in the United States. In EPA's Health Effects Division and for pesticides in general, a substantial amount of data based on chemical use is required to register a pesticide. These data requirements include details from studies on acute toxicity, subchronic toxicity, developmental toxicology, reproductive toxicology, carcinogenicity, and neurotoxicology. Because the FQPA mandated the screening for EDCs, the Office of Science Coordination and Policy has spent considerable time and effort over the past 10 years to look for, evaluate, and use information on EDCs in risk assessments for pesticides. To this end, a tiered approach emerged for the EDSP. Tier 1 is focused on the screening assays, whereas Tier 2 addresses the issue of functional endpoints of concern for EDCs. In aiming to implement the work that has evolved in the EDC arena, the first challenge is to examine assay data sets that have come forward through this EDSTAC process, and to compare that information with existing data sets to see what matches can be found. From a national and global perspective, finding the best data set is critical to permit registration of the chemicals.

Dana Heriegel

Ms. Dana Heriegel spoke from the policy perspective, giving an overview of the organization and focus of the regional offices. EPA has 10 regional offices nationwide, with Region 3 including the States of Delaware, Pennsylvania, Massachusetts, Virginia, West Virginia, and the District of Columbia. In Ms. Heriegel's office, topics of interest include the public perception of water quality, how to handle this viewpoint, and what details are involved with an unwanted chemical collection event. Region 3 is receiving guidance on public perception from Regions 1 and 5, which have experience handling issues regarding public viewpoints. Another focal area is on pollution prevention mechanisms to prevent contamination before it gets to the water and keeping it out. An example of this topic is to note whether medication used at a particular in-house pharmacy in a hospital is meeting the right criteria in terms of disposal and discharge details. Ms. Heriegel also emphasized the importance of communicating research findings to the regions and program offices. This information then is communicated to the states and along to the drinking water and treatment facilities to broaden the existing knowledge base.

General Discussion

Dr. Laessig introduced three questions to frame the discussion:

- Are the exposures and endpoints being studied appropriate and useful for regulators and risk assessors?
- How will the methods and models being developed be used and by whom?
- How can ORD improve communications with programs, regions, and the scientific community about EDCs research?

Dr. Laessig opened the discussion by asking the panelists to describe what, if anything, has piqued their interest from today's session that will be of use to them.

Ms. Eignor commented that she is very interested in the details that Dr. LeBlanc mentioned about nutrients. She currently is on EPA's National Nutrient Team, and there will be a push next year to have

the states develop nutrient standards. Ms. Eignor pointed out that although criteria can always be developed, the legislature must adopt them. Her office is spending a considerable amount of time with the regions to help them develop nutrient criteria.

Mr. Timm stated that it was useful to hear about research on metals because the usual focus with EDCs is on organic materials; it helps to have a broader perspective. He added that it was useful to hear about pharmaceuticals and personal care products because there are many substances, besides pesticides, to which people are exposed and that might pose a concern. Mr. Timm also found work on the mixtures work interesting and echoed earlier comments that, traditionally, one chemical is regulated at a time, whereas combined chemical effects are of less focus. He pointed out that the FQPA has accounted for this growing need by mandating that, in addition to having a screening program, cumulative risk should be examined as well.

Dr. Scarano explained that part of what was mandated by the FQPA was for pesticides research to consider aggregate and cumulative exposure. For aggregate exposure, different groups of exposures would be analyzed and then combined to provide some type of estimate of risk associated with different sources. For cumulative exposure, information on exposure and hazards would be pooled for chemicals with common MOAs to provide a risk assessment. Dr. Scarano mentioned that epidemiological studies are of great personal interest to him.

Dr. Laessig commented that the STAR Program has been trying to initiate observational studies, with one research direction centered on pesticides and children; however, it has been difficult to obtain approvals for these types of studies.

Mr. Timm pointed out that a rather solid basis of EDC effects in wildlife exists, through field confirmation of observations made in the laboratory. The evidence is weak on the human side, however, and this supports the need for epidemiological studies to make critical linkages.

Dr. Anderson noted that metabolites are present in the environment but have not been studied very much. Consequently, they are difficult to regulate. He asked if there is any plan in place to regulate metabolites. Likewise, is there any regulation in place to govern mixtures? He added the comment that it would be helpful for scientists to be kept abreast of chemicals that are of regulatory interest in light of the lengthy timeframe associated with epidemiological studies.

On the question of regulating metabolites, Mr. Timm commented that the SDWAA gave discretionary authority to use the screening program to address contaminants that are neither manufactured products nor pesticides. As the program matures, some of this authority must be exercised and extended to the issue of metabolites.

There has been some progress on the metabolite front, Dr. Scarano noted. For instance, recently it has been discovered that the conozole group of chemicals has a single common metabolite—1,2,4-triazole. This prompted a group of product registrants to get together and generate some information that will be of use during risk management considerations.

Ms. Eignor commented from her past experience with EPA's Office of Pesticide Programs that, if she was concerned about a metabolite, she had the right to request a report from the product registrant. Regarding funding, she added that the Office of Water is being scrutinized heavily with respect to grants. EPA now is focusing on other funding vehicles, including interagency agreements, cooperative agreements or contracts, and working with universities.

Dr. Swan suggested that epidemiological studies would benefit from more environmentally relevant dosing and routes by which humans are exposed to EDCs. In this light, funding studies on rodents and other animals that experience exposures resembling those of humans is valuable.

Mr. Timm commented that more research is needed to clarify the issue of low dosage exposures to EDCs. Once this is understood better, more environmentally relevant concentrations could be introduced in toxicological testing.

Dr. Laessig mentioned that she has spoken with program and regional representatives during relevancy reviews and learned that they value ecotoxicology work. She asked the panelists if this also is of value to them.

Ms. Eignor commented that she is more of an ecological person who does human-related work on the side. Mr. Timm noted that although the main focus of EPA's EDSP is the LTG 3 of developing assays, LTGs 1 and 2 are equally important because a thorough understanding of a risk is necessary before an effective and intelligent risk assessment is possible.

Dr. Laessig thanked the panelists for their comments and adjourned the panel discussion of Session I at 5:30 p.m.

SESSION IV: THYROID TOXICANTS—EXPOSURE AND EFFECTS ON BRAIN DEVELOPMENT AND DISEASE

Session Introduction
Mary Gilbert, EPA/ORD/NHEERL

The second day of the workshop began with an introduction to thyroid toxicants by Dr. Mary Gilbert on some ongoing research in the Neurotoxicology Division. Three STAR grantees followed, covering the topic of TH disruption via exposures and outcomes in human populations and animal models.

Thyroid Toxicants: Developmental Outcomes and Chemical Mixtures
Mary Gilbert, EPA/ORD/NHEERL

Thyroid hormone (TH) is essential for normal brain growth and development. To relate exposures to thyroid disrupting chemicals and clinical disease in human populations, a biologically based dose-response model has been adopted. This model considers various targets and mechanisms by which the TH axis can be disrupted and provides a link to neurological impairments such as hearing loss and learning deficits. Serum TH levels are used as an index of perturbation of the thyroid axis. One way in which circulating levels of serum TH can be reduced is by increasing hormone metabolism and elimination. Various environmental contaminants induce glucuronidation of T4 and these chemicals display distinct dose-response profiles. One NHEERL project has found that adding these chemicals together in a complex mixture will reduce the serum level of TH, as predicted by the additivity theory, although the effects are underestimated at high doses. Another project is developing *in vitro* assays using hepatocytes from mouse, rodent, and human to find the most appropriate model to improve cross-species extrapolation. Other studies are linking the degree of thyroid disruption with neurodevelopment and have shown that subclinical hormone reductions in pregnant women result in IQ deficits in their offspring. Critical factors that impact the outcome are the magnitude, timing, and duration of the TH insufficiency. For instance, prenatal hypothyroidism produces cortical malformations. Postnatally, correlations have been demonstrated between serum T4 and hearing loss. To investigate these findings further, behavioral and cellular models of cognitive endpoints have been developed. One study has demonstrated a dose-dependent reduction in hippocampal synaptic transmission in animals exposed to propylthiouracil (PTU).

Hippocampal learning also has been harmed, as has been shown by the impairment of Morris water maze acquisition and reversal learning in adult offspring. The information acquired from the various studies on TH disruption will provide the platform on which to derive computational models predictive of the effects of thyroid toxicants on neurological development.

Endocrine Disrupting Chemicals and Thyroid Outcomes
Henry Anderson, Wisconsin Department of Health and Family Services

Research was conducted to assess associations between fish consumption, contaminant levels, and thyroid and reproductive hormone function in men and women living in the Great Lakes (GL) Basin. The study measured PCB, dichlorodiphenyldichloroethylene (DDE), and polybrominated diethyl ether (PBDE) serum concentrations and endocrine health status by analysis of blood and urine specimens and survey responses to health status questions. This study enrolled participants from an earlier study in 1993–1994 on GL fish consumption that surveyed nearly 4,400 GL charter boat captains, anglers, and infrequent sport fish consumers and their wives living in Illinois, Indiana, Michigan, Ohio, and Wisconsin. In 1995–1996, many of the earlier cohort participants were recruited to participate in a study measuring PCB, DDE, dioxin, and furan exposure; birth outcomes; and reproductive and TH status. In 2002–2004, those with previous serum organochlorine determinations were invited to participate in a longitudinal biomarker study to examine trends over time. A 10-page questionnaire on current health status and fish consumption was mailed in 2004 to 3,865 of the original cohort members; nearly one-half of the surveys were returned in completion. For 531 participants who completed the survey, a second and more detailed survey involving additional questionnaires and blood and urine collection was conducted. Preliminary assessments comparing the two surveys revealed a decline in the number of sportfish GL meals per year for both men and women. Based on 350 to 363 participants' blood results for environmental contaminants, the average PCB, DDE, and PBDE levels in μg/L were 3.0, 2.8, and 0.9, respectively. Based on 85 paired samples (46 men, 39 women), PCB and DDE values have decreased from 1993 to 2005. Average thyroid levels in 1994, including both men and women and excluding those on thyroid medications or other drugs affecting thyroid function, were 118.8 ng/dL for T3, 7.0 μg/dL for T4, and 1.9 μIU/mL for TSH. For 2005, the average levels (not excluding participants) were 98.8 ng/dL for T3, 7.6 μg/dL for T4, and 1.9 μIU/mL for TSH. Upcoming work will involve completion of all remaining laboratory analyses by the end of 2006, with results letters sent to the participants.

Discussion

Dr. Ferguson asked to what extent were the measurements of TSH elevated or depressed out of range, and what would the figure be for the control group. A participant from Dr. Anderson's group responded that TSH levels were mostly elevated. Dr. Anderson added that people who are on medications need to be removed from the analysis for thyroid function. Patterns in the results are still being assessed for these thyroid data.

Dr. Francis asked what information is being provided back to the participants in the results letters and to what extent do participants want to know what the results mean. Dr. Anderson responded that most people indicated on the consent form that they wanted to learn their results. A member of Dr. Anderson's group added that the participants are given information on PCBs and DDE but not on PBDEs because there is no general consensus on results for this chemical and the group does not have information from previous studies. For the PCBs and DDEs, the participants are given their values, along with the average values for men and women, for comparison sake. For the thyroid information, they are given the range to which they can compare their values. It also is suggested that the participants can show their values to their physicians.

A participant noted that the correlation between serum lipid levels and age explains why a fairly predictable pattern results from the way that some pesticides have an age effect when adjusted for lipid levels. He suggested that future discussions take into account such details regarding bioavailability. Dr. Anderson responded that the variability in the population is no longer what it used to be because many people are on medications, such as Lipitor, which effectively removes the measurement of people in the high lipid range.

Low Dose Effects of Thyroid Toxicants on Neurodevelopment
Thomas Zoeller, University of Massachusetts, Amherst

Adaptive responses of the thyroid system to low-dose perturbations by thyroid toxicants are a focus in this study. As these toxicants can act by different MOAs, different profiles of changes in hormone levels, thyroid histology, and effects on brain development can result. This study hypothesized that the adaptive responses of the thyroid system are differentially effective when induced by toxicants with different MOAs. A specific aim is to determine the relationship between TH dose and response of several developmentally important endpoints in the brain, liver, and heart. Endpoints of TH action will include the expression of genes known to be TH-responsive in the development of the brain, liver, and heart. Developmental endpoints will include measurements within portions of the brain, and size and weight of the liver and heart. The work is focused on three toxicants: PTU, perchlorate, and PBDE. Pregnant rats were treated and their pups were sacrificed on PND 15 for analyses. The study has found that PTU treatment reduces serum T4 levels in the pups but does not alter body weight. Moreover, TH increases the number of oligodendrocytes and decreases the number of astrocytes. The dose of PTU required to reduce T4 is lower than the dose of PTU needed to affect white matter. The effects of perchlorate exposure in drinking water revealed significant increases in serum thyroxine binding globulin in PND 15 rats at low, environmentally relevant levels of perchlorate. Work in progress includes the completion of serum and tissue hormone analysis in PTU and perchlorate experiments, and completion of the tissue analysis of TH endpoints. A future step is to run the PBDE experiment.

Discussion

A participant commented that many of the measurements were taken on the postnatal offspring. What proportion of the dose got transferred prenatally or was the majority of their effect coming from food the pups ate on their own? Dr. Zoeller responded that neither PTU nor perchlorate was provided in the food, so it is unlikely that the pups would be receiving those chemicals directly; they were most likely received lactationally. The same participant asked whether the perchlorate is transferred in the milk. Dr. Zoeller confirmed that the chemical is being transferred in milk. He does not think it is being transferred in the drinking water.

Development of a Biologically Based Pharmacokinetic (BBPK) Model for the Hypothalamic-Pituitary Thyroid Axis in the Maturing Rat for the Dose Response Assessment of Developmental Neurotoxicity
Jeffrey Fisher and Duncan Ferguson, University of Georgia

This research aims to develop biologically based models of the hypothalamic-pituitary thyroid (HPT) axis for different reproductive states of rats and to couple the HPT axis with physiologically based pharmacokinetic models for thyroid-active chemicals that disturb the axis. The ultimate goal is to be able to define a dose-response relationship via prediction of developmental neurotoxicity based on degree of expression of disturbances in the HPT axis. Current work is focused on developing radiolabel submodels for iodide and T4 in PND 13 and the adult rat; examining feedback equations for endogenous serum TSH and T4 concentrations; and developing an iodide model for the adult human. The experimental work is using PTU as a prototypical compound to establish both high dose- and low-dose response relationships between disruption of the HPT axis leading to hypothyroidism in the developing rat and resulting in

neurotoxicity. Rats were treated with 0, 3, and 10 ppm PTU in the drinking water from gestational day 2 through PND 21–30. When the male pup rats and dams were sacrificed, serum was collected, and various tissue samples were taken. Adults were sacrificed starting 2 months after weaning, at an average of PND 100. At PND 21, the pups were assessed for electrophysiology. Results have shown that hepatic type I 5'-deiodinase (D1) fell in a dose-dependent manner, with a greater percentage effect on the pups than dams at PND 21–30. In contrast, cerebrocortical type II 5'-deiodinase enzyme (D2) increased exponentially in both pups and dams to maintain the brain T3 concentrations, with the pups experiencing a maximum level at the 3 ppm dose. The cortical T3 levels remained normal until the serum T4 concentration fell 75 percent. Assessments at PND 100 revealed normal thyroid, cortical T3, and D1 and D2 activities. Results for electrophysiology assessment revealed a strong and positive association between D2 activity and hippocampal synaptic potential at PND 21–30. Upcoming work will include examination of additional tissue thyroid markers and refining the dose studies down to the 0.3 ppm level.

Discussion

A participant commented that most of the correlations for biomarker development were taken at a single time point. She asked whether there is a plan to do profiles across time. Dr. Ferguson responded that his group is conducting a study now that includes dosing at PND 5, 15, 21, and in the adult. The same participant commented that the model structure shown by Dr. Fisher did not include the active transport of iodide and perchlorate in the skin that was used by the studies referenced in his presentation (Clewell, et al., 2003; Yu, et al., 2002). She asked whether the current study plans to address this detail. Dr. Fisher responded that his team is fitting the data without this aspect for pups younger than PND 14, because at that delicate stage there might be difficulties with handling the skin.

Another participant asked what consequences of the electrophysiology persisted. Dr. Gilbert responded that her group has done electrophysiological assessments in a different part of the hippocampus. The plasticity model her group is using is based on a cellular model and the expectation would be that if there is a reduction in the capacity for the system to support plasticity in the hippocampus, some behavioral deficits that are mediated through the hippocampus should be observed. Dr. Gilbert added that her presentation gave examples of impaired hippocampal learning, including a reduction in the ability of the animal to perform a spatial task upon PTU exposure.

A participant noted that Dr. Fisher's team is using PCB126 as one of the prototype chemicals, and that it is an age-receptor activator, which binds the nuclear receptor and affects THs by altering metabolism. He added that there are many other chemicals that alter other nuclear receptors, including binding associated with peroxisome proliferator-activated receptor (PPAR). How applicable is the model structure for PCB126 to those chemicals? Dr. Fisher responded that his team's modeling approach is based primarily on some aspects of dioxin work and that they have age-receptor activation at the onset. He explained that the approach could be used for other nuclear receptors as long as binding constant information is available. The participant added that the equations would stay the same and only the parameters would be changed. So, although PCB126 may be a prototype for a dioxin, the model structure would actually be applicable to any chemical that activates nuclear receptors. Dr. Fisher agreed with this statement, adding that the liver is important for many chemicals through nuclear receptor activation. The participant mentioned that it also allows us to think about the dose-response relationship and how to extrapolate that from animals to humans.

SESSION V: ANALYTICAL METHODS FOR EDC MIXTURES

Session Introduction
Bryan Boulanger, EPA/ORD/NRMRL

Dr. Bryan Boulanger welcomed the participants and provided an overview of this session. The presentations in this session gave examples of novel analytical techniques for addressing the problem of measuring cumulative endocrine activity in environmental samples.

U.S. EPA's Risk Management on EDCs
Bryan Boulanger, EPA/ORD/NRMRL

Risk management (RM) research on EDCs examines the major sources and environmental fates of these chemicals and how unreasonable risks can be controlled. EPA's National Risk Management Research Laboratory (NRMRL) is working on research examining EDCs emitted from WWTPs, CAFOs, and present in drinking water. The WWTP research ranges from analytical methods development all the way to pilot plant research. Methods development includes such research as adapting bioassays for use in the field. An example of bench-scale research is a project evaluating the fate of alkylphenols and steroid hormones under conditions common to wastewater treatment and sediments, including aerobic, anaerobic, sulfate reducing, and methanogenic conditions. Field-scale WWTP research includes the North Carolina Study, which is examining the fate of anaerobically digested and lightly limed biosolids that were applied to a fescue field in September 2004. On the animal side, CAFO RM research aims to improve understanding of waste concentrations, examine fate and transport of wastes in soils, and assess the efficacy of CAFO waste management practices. CAFO contributions of estrogens include cattle, poultry, and swine; among these, only cattle are given growth-enhancing hormones. Currently, limited field data are available to show that CAFOs can contaminate ground and surface waters with estrogens, and long-term fate studies are needed at each site. Drinking water research includes methods development for estrogenic and androgenic hormones and bioassay development for drinking water treatment operations. Future research will include work on tertiary wastewater treatment; expanded characterization of CAFO sources; and development of a full pilot-scale data set for drinking water research.

Discussion

Dr. Anderson asked if any long-term monitoring programs will address whether EDCs in waste streams are increasing or staying the same. Dr. Boulanger responded that if this type of monitoring system is to be set up, it probably will occur through EPA's NRMRL, in the exposure research laboratory. Regarding the cattle, Dr. Boulanger explained that implants are placed beneath the skin of the ear that release hormones to the animals. Research is needed on the different types of implants to see which ones are contributing more chemicals. It is not expected that trenbolone would be detected in the waste streams, he added, because this chemical is phased out through the dosing process for the animals.

Dr. Lazorchak made mention of a study that collected water samples from a cattle CAFO through four seasons to measure trenbolone levels. Although the levels are seasonal, the chemical should not be detected in the receiving water of CAFOs at any time. It is of interest to determine the duration of detection during the year.

Integrated Microfluidic System for Bioluminescent Bioreporting, Separations, Vibrational Spectroscopy, and Microcantilever Transducer Evaluation of EDCs
Michael Sepaniak, University of Tennessee

The goal of this project is to develop a fully integrated microfluidic device for the detection of EDCs. It is

envisioned that one platform will house several operations, including bioluminescent reporters of EDC presence, separations, and quantitative vibrational and nanochemical detection of EDC-containing samples of known and unknown composition in specially created matrices. The analytical methodology components of the platform are surface-enhanced Raman spectroscopy (SERS) substrates being optimized specifically for EDCs; microcantilever arrays (MCAs) functionalized with molecular recognition phases; and running buffer conditions to permit separation of select mixtures of EDCs via electrophoretic methods. Channels created in PDMS films using photolithography appear on the transducer chip, which measures in μm roughly 50 (wide) by 200 (long) by 1 (thick). To permit increased responsiveness to a range of analytes, surface nanostructured and chemically functionalized MCAs are confined within the platform's microchannels. Following initial exposure to an analyte, the MC tranducers undergo bending in response to protein-protein interactions and charge effects. This bending is monitored via optical bending technique such that the vertical cavity surface emitting lasers are reflected from the tips of the MCs onto a position detector. An MCA can have 10 or more cantilevers, each with a different bioaffinity coating, permitting the simultaneous testing of several chemicals. Initial separations have been performed using a conventional capillary electrophoresis instrument to determine the required buffer conditions. Thus far, the SERS activities of about 12 EDCs have been assessed using the random morphology nanocomposite substrates. Distinctive SERS spectra of EDCs using Ag-PDMS substrates have included results for bisphenol A, kaempferol, and apigenin. Over the next year, work will include MCA modifications to enhance responses and selectivity, developing conditions for on-chip separations for various target EDC combinations, and working toward integrating the individual analytical components and field portability.

Discussion

Dr. Boulanger asked for a cost estimate on the equipment. Dr. Sepaniak gave an estimate of $5,000. He stated that his group builds the position sensitive detector, and the lasers could be as simple and inexpensive as the laser pointer used in presentations.

Dr. LeBlanc stated that it seems that the major limitation for the various methods is that analytes need to be handled at the micromolar range. Dr. Sepaniak confirmed this point and added that EDC exposure levels are down to the 10^{-8} molar level. To improve on sensitivity of the analysis, the thickness of the cantilevers can be modified.

Dr. Lazorchak asked what sample volume is used for this work. Dr. Sepaniak responded that the sample volume is about 10 nL, with the entire filled channel housing approximately 1 μL. Dr. Lazorchak asked if the methodology can run bigger samples instead of concentrating the material into small volumes. Dr. Sepaniak responded that larger samples can be run via controlled delivery of the analyte to the surface. By exposing the sample to more mass or volume, the material will accumulate on the surface of the SERS substrate.

Dr. LeBlanc commented that, ultimately, this technique would have environmental application. He asked if there is concern about organic material in the environment interfering with the sampling regime. Dr. Sepaniak responded that this is why separation is a critical step—you detect only what you are interested in. He added that the technologies have field-ready potential such as via miniaturization of the MCA components.

Dr. Laessig asked whether the technique can be applied to serum samples. Dr. Sepaniak replied that this is not currently an intended application.

A participant asked whether there has to be any type of blocking on the cantilever surface to obtain a certain type of deposition. Dr. Sepaniak explained that sometimes blocking is necessary and that mobility

is an issue. Having the mobilized species retain all of its activity may not necessarily be desirable. For instance, when protein A was used to orient the antibodies, worse responses resulted.

Development of Receptor to Population-Level Analytical Tools for Addressing EDC Exposure in Wastewater-Impacted Estuarine Systems
Lee Ferguson, University of South Carolina

Assessment of EDCs in complex mixtures is the focus of this study, which seeks to develop sensitive, MOA-based bioanalytical tools for detecting EDCs in environmental mixtures, such as wastewater. The approach will involve development of nuclear hormone receptor-affinity extraction techniques as tools for isolating EDCs from complex wastewater mixtures. Recombinant nuclear hormone receptors, including estrogen, androgen, TH, and ecdysteroid/ultraspiracle, are being used to construct bioaffinity-extraction columns. The system uses an eluent to remove the EDCs from the sample. The material then is analyzed using quantitative HPLC-MS/MS, HPLC/Q-TOF MS/MS, and GC-MS. Work in progress includes the preparation and characterization of nuclear hormone receptors for preparation of receptor affinity columns. The ER-α ligand-binding domain already has been cloned and expressed in bacterial vectors, and the team currently is validating binding affinity using a radioligand assay. Recent work has begun on cloning the human androgen receptor ligand-binding protein into an expression vector. Material for the testing will originate from two sites representing wastewater discharge regimes typical of the South Carolina coast—Plum Island WWTP and Kiawah Island WWTP. To link EDC exposure to biological effects, the *in vivo* activity of receptor-isolated EDCs will be examined through vertebrate (zebrafish) and invertebrate (copepod) assays. Upon EDC exposure, the zebrafish are assayed for vitellogenin. The copepod bioassay exposes the microcrustaceans to EDCs in a microplate, with reproductive and developmental measures as endpoints. Upcoming work will include initial immobilization studies and validation with simple EDC mixtures and with the complex environmental samples collected from the WWTP locations.

Discussion

Dr. LeBlanc commented that the proof-of-concept work with affinity chromatography used antibody as opposed to receptor, and that the antibody results were quite resilient. He asked whether the receptors will be as tough. Dr. Ferguson responded that there has been some work by a group in France that used ER-α ligand-binding domain to do similar binding work; in their case, however, only about five cycles were generated from their column. With its antibody columns, the present study could go up to 30 cycles. To validate the receptors, Dr. Ferguson's study is using a surface plasma resonance system to help maintain the stability of the receptors, using the same binding chemistry as is used for the immobilization step. Thus far, the surface has been regenerated nicely on the system; it is hoped the results can be translated to the surface of the beads in the affinity column.

Regarding the EcR used in the affinity approach, Dr. LeBlanc commented that this step involves the dimer, EcR/USP. He asked if these two components will be covalently linked. Dr. Ferguson responded that the plan is to covalently attach them to the surface, but not to each other. Dr. LeBlanc sought clarification, asking whether they will find each other if they are covalently attached to the surface. Dr. Ferguson replied that that he is going to incubate them first to allow them to dimerize with the ligand, and then elude the ligand off.

Dr. Francis asked how the study plans to follow up on its aim to take the study data from the copepod to the population level. Dr. Thomas Chandler responded that the plan is to take the laboratory assay results and apply it to field populations. The salt marshes along Kiawah Island have some of the richest coastal populations of copepods on the East Coast. The study then will try to validate the field data in the laboratory.

Dr. Francis asked whether the methodologies used for wastewater also are applicable to effluent from CAFOs. Dr. Ferguson explained that the mixture cannot be too complex when it is added to the bioaffinity column. A solid-phase extraction step must first occur. Depending on the complexity of the upstream sample, however, the downstream steps of the affinity column should be applicable to any type of aqueous sample. He pointed out that CAFO effluent is more similar to sewage influent, which tends to be very complex and of high concentration. Thus far, the team has had good success using the immunoaffinity columns from sewage influent from a WWTP when the samples were diluted properly.

Workshop Summary
Susan Laessig, EPA/ORD/NCER

Dr. Laessig summarized the main points of the meeting. The primary outcomes included integrating data from studies on related topics; comparing outcomes from animal and human studies; creating and strengthening collaborations; and learning how risk research is used for risk management, regulations, and policy decisions. From Session I, participants learned that there is global concern regarding the effects of EDCs on reproduction, as shown from animal studies and associations in wildlife. Various studies also are demonstrating EDC effects in humans. More work is needed on determining causality of effects and examining how to approach the observed effects. Among Session II's topics relating to the effects of EDCs on puberty were animal models for human disease, screening assays, and a look at dose-response and mechanisms. Session III covered the area of biomarkers of exposure to EDCs, broaching such topics as linking exposure to effects, field applications, and new approaches to cumulative exposure assessment. Thyroid toxicants and their effects on brain development and disease were covered in Session IV, which included the topic of fish consumption and effects in humans. Session V focused on analytic methods for EDC mixtures and featured presentations discussing risk management, combined use of analytical and bioassay methods, and examination of complex mixtures.

The panel discussion during Day 1 of the meeting emphasized the importance of communicating the relevance of research to EPA, identifying research needs, and developing new outreach methods. At the close of Day 1, a poster session provided an opportunity for graduate students and postdoctoral researchers to benefit from informal discussions of their work.

Dr. Laessig remarked that the series of presentations was a success and thanked everyone for their participation, including the volunteers, the grantees, the program offices and regions, and the session chairs. She adjourned the meeting at 2:50 p.m.

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2006 STAR Progress Review Workshop

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Participants List

Henry Anderson
Wisconsin Department of Health and Family Services

Willard Anderson
U.S. Environmental Protection Agency

Dean Baker
University of California, Irvine

Ruby Bansal
University of Massachusetts, Amherst

Don Bergfelt
U.S. Environmental Protection Agency

Linda Birnbaum
U.S. Environmental Protection Agency

Chad Blystone
North Carolina State University

Kathy Bobseine
U.S. Environmental Protection Agency

Bryan Boulanger
U.S. Environmental Protection Agency

Michael Breen
U.S. Environmental Protection Agency

Jane Caldwell
U.S. Environmental Protection Agency

Tom Chandler
University of South Carolina

Pei-Jen Chen
U.S. Environmental Protection Agency

Glinda Cooper
U.S. Environmental Protection Agency

Geraldine Cripe
U.S. Environmental Protection Agency

Kevin Crofton
U.S. Environmental Protection Agency

Sally Darney
U.S. Environmental Protection Agency

Mike DeVito
U.S. Environmental Protection Agency

Susan Euling
U.S. Environmental Protection Agency

Sue Fenton
U.S. Environmental Protection Agency

Duncan Ferguson
University of Georgia

Holly Ferguson
U.S. Environmental Protection Agency

Lee Ferguson
University of South Carolina

Jeff Fisher
University of Georgia

Paul Foster
National Institutes of Health

Jack Fowle
U.S. Environmental Protection Agency

Elaine Francis
U.S. Environmental Protection Agency

Michael Gage
U.S. Environmental Protection Agency

Brent Gilbert
North Carolina State University

Mary Gilbert
U.S. Environmental Protection Agency

Susan Glassmeyer
U.S. Environmental Protection Agency

Barbara Glenn
U.S. Environmental Protection Agency

Michael-Rock Goldsmith
U.S. Environmental Protection Agency

Kimberly Gray
National Institutes of Health

Soon Young Han
U.S. Environmental Protection Agency

Russ Hauser
Harvard School of Public Health

Jerry Heindel
National Institutes of Health

Dana Heriegel
U.S. Environmental Protection Agency

Ross Highsmith
U.S. Environmental Protection Agency

Victoria Holt
Fred Hutchinson Cancer Research Center

Andrew Hotchkiss
U.S. Environmental Protection Agency

Michelle Hotchkiss
U.S. Environmental Protection Agency

Keith Houck
U.S. Environmental Protection Agency

Pamela Imm
Wisconsin Department of Health and Family Services

Sung Jae Kim
U.S. Environmental Protection Agency

Adam Kuhl
CIIT Centers for Health Research

Susan Laessig
U.S. Environmental Protection Agency

Susan Laws
U.S. Environmental Protection Agency

Jim Lazorchak
U.S. Environmental Protection Agency

Gerald LeBlanc
North Carolina State University

Hong Li
North Carolina State University

Susan Makris
U.S. Environmental Protection Agency

Mary Beth Martin
Georgetown University

Edward Massaro
U.S. Environmental Protection Agency

Michael McClure
U.S. Environmental Protection Agency

Eva McLanahan
University of Georgia

Leonard Mole
U.S. Environmental Protection Agency

Debdas Mukerjee
U.S. Environmental Protection Agency

Libby Myers
University of Georgia

Nadia Paolino
University of Georgia

Jim Raymer
RTI International

Jennifer Rayner
U.S. Environmental Protection Agency

Bethany Reeves
North Carolina State University

Vicki Richardson
U.S. Environmental Protection Agency

Louis Scarano
U.S. Environmental Protection Agency

Paul Schlosser
U.S. Environmental Protection Agency

Heiko Schoenfuss
Saint Cloud State University

Bono Sen
U.S. Environmental Protection Agency

Michael Sepaniak
University of Tennessee

David Sharlin
University of Massachusetts

Carol Sloan
RTI International

Tammy Stoker
U.S. Environmental Protection Agency

Michael Stramiello
University of Georgia

William Studabaker
RTI International

Shanna Swan
University of Rochester School of Medicine

David Szabo
University of North Carolina

Matthew Taylor
University of Georgia

Daniel Tighe
University of Massachusetts, Amherst

Nicolle Tulve
U.S. Environmental Protection Agency

Helen Wang
North Carolina State University

Stephen Watkins
U.S. Environmental Protection Agency

Sally White
U.S. Environmental Protection Agency

Christopher Zarba
U.S. Environmental Protection Agency

Robert Zoeller
University of Massachusetts, Amherst

Contractor Support

John Hare
The Scientific Consulting Group, Inc.

Deborah Komlos
The Scientific Consulting Group, Inc.