

e.hormone 2009 poster abstracts

1.

Eutrophication is Environmental Obesity And Other Stories of Excess

Thea M. Edwards

Tulane University, New Orleans, LA 70118

It is a truth universally acknowledged, that an organism in possession of good fortune, must be in want of nutrients. Jane Austin's wry sense of humor signals that this is but a superficial expectation. Over evolutionary time, animals and ecosystems have evolved to detect, manage, or sequester those valuable molecules that promote fitness and limit disease: sugars, nitrates, folic acid, cholesterol, estrogens are just a few. In nature, these molecules are limited and therefore valuable and powerful. So much so, that programming to gainfully respond to these molecules begins in the embryo. With the arrival of human ingenuity, sugar cane cultivation, the Haber-Bosch process, vitamins in a bottle, big macs, and bisphenol A, those recently limited systems became flooded with signals and substrates. This flooding causes overgrowth and disease, and rapidly changes the physiological context for cells, organisms, and ecosystems. The effects of excess in systems adapted for leaner times can be understood within the framework of the "Predictive Adaptive Response Hypothesis", an outgrowth of the "Thrifty Phenotype Hypothesis". I argue that this concept can be extended well beyond the caloric-restriction-obesity paradigm to generally improve predictions about excess.

tedwards@ufl.edu

Phone: 504-862-8454

Fax: 504-988-6428

2.

Environmental Activism at Music Festivals

Anna Whalen

Tulane University
Department of Environmental Studies

Many popular American music festivals are attempting to be environmentally sustainable and are promoting themselves as “green” events. This study examines the disparities between the rhetoric and the implementation of environmental programs at music festivals. Seven music festivals were attended in various regions of the United States. Information was gathered about the energy sources used to power festival operations, including whether or not they include the use of solar panels and bio-fuels. Waste management policies were investigated, focusing on the types and amounts of waste produced, and on the processes used to sort waste into composting, recycling, and landfill. This study found that the efficacy of sustainability efforts varied among festivals. Factors that were relevant include: festival commitment and organization, regional location, sponsors, festival layout, convenience of waste stations, availability of potable water, vendors, environmental activist representatives, and audience behavior. Music festivals provide a framework for exposing the environmental standards prevalent throughout the United States. They illustrate problems with public understanding of environmental issues and of the divergence between policy and practice. Festivals also illuminate strategies that can facilitate environmentally responsible public action and effective waste management.

Email: whalen.anna@gmail.com

Phone: (510) 220-7881

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3.

The Evolution of Estrogenicity: How Has the Ligand Evolved?

Robert Wallace, Bo Han, Michelle Lacey, and John McLachlan

Tulane/Xavier Center for Bioenvironmental Research, New Orleans Louisiana

Remarkable progress has been made in the last few years in tracing the evolution of the Estrogen Receptor (ER) and other members of the Nuclear Receptor (NR) family. In addition, the details of Fast Signaling by Estrogen have become clearer. There remains an intriguing problem on which virtually no progress has been made—Why do so many organisms make chemicals which can mimic estrogen? Organisms as diverse as fungi, turmeric, and tunicates make estrogenic chemicals, and organisms as diverse as bacteria and nematodes respond to estrogenic chemicals. This question is less tractable than that of the evolution of receptors, in part because estrogenic chemicals are the products of synthetic pathways, and so comparing protein or genetic sequences to infer evolution is not possible. With the general hypotheses that some ancestral estrogen-like molecule was one of the earliest signals for differentiation, and that this molecule worked before there was a Nuclear Receptor to bind with it, we propose to investigate the question in three ways. 1) What specific features of molecules confer estrogenicity?: 2) Are there homologies in the synthetic pathways of estrogenic molecules that can be used to reconstruct an evolutionary history?; 3) Are there homologies in the non-ER signaling pathways that can be used to reconstruct an evolutionary history? We have some results back on the first question. In a cluster analysis of all 135 estrogenic compounds listed in PubChem we found that all the estrogens from plants and fungi fall in one cluster. We have begun work on the two other questions by searching genomic databases with BLAST for enzymes in synthetic pathways and for tyrosine kinase receptors.

Presenting Author: Robert Wallace, rwallace@tulane.edu, (504) 988-6512

4.

Umbilical Cord Serum Organochlorines and Child Language Development: a Cohort Study

Scott A. Venners¹, Glenys M. Webster², Sally W. Thurston³, Changzhong Chen⁴, Susan Korrick^{4,5}

1. Simon Fraser University, Faculty of Health Sciences, Burnaby, British Columbia, Canada;
2. University of British Columbia, School of Environmental Health, Vancouver, British Columbia, Canada;
3. University of Rochester, Department of Biostatistics and Computational Biology, Rochester, New York, USA;
4. Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA;
5. Department of Environmental Health, Harvard School of Public Health, Boston, Massachusetts, USA

Knowledge of how organochlorine exposures affect cognitive development is limited. We measured 51 congeners of polychlorinated biphenyls (PCB's), the p,p' isomer of dichlorodiphenyldichloro-ethylene (p,p'-DDE), and hexachlorobenzene (HCB) in umbilical cord serum of 604 newborns whose mothers took part in a birth cohort in Massachusetts near a PCB contaminated harbor and Superfund site. Fifth, 50th and 95th percentile cord serum concentrations (in ng/g serum) were p,p'-DDE: 0.11, 0.31, 1.3; HCB: 0.007, 0.023, 0.053; and sum of four most prevalent PCB's (sum(PCB)): 0.06, 0.19, 0.62. When the children were 7 to 10 years old, we administered the Boston Naming Test, a confrontational naming instrument in which subjects name objects pictured in line drawings. The median number of spontaneous correct answers was 35 (5th and 95th percentiles: 23 and 44). Among 570 mother-child pairs with complete covariate data, sum(PCB) was not associated with spontaneous correct answers after covariate adjustment. Children at the 95th percentile of log(p,p'-DDE) had 1.6 (SE=0.8, p=0.04) fewer spontaneous correct answers on average than children at the 5th percentile after covariate adjustment, whereas for log(HCB) the children with 95th percentile exposure had on average 1.4 (SE=0.7, p=0.05) fewer spontaneous correct answers than children at the 5th percentile. These associations were not affected by adjustment for sum(PCB). All three exposure measures were significantly positively correlated (p=0.001). When log(p,p'-DDE) and log (HCB) were modeled together, adjusted effect estimates were attenuated: log(p,p'-DDE) ($\beta = -1.2$, SE=0.8, p=.129) and log (HCB) ($\beta = -1.1$, SE=0.7, p=.152).

Presenting author information:

Scott A. Venners, Assistant Professor

Simon Fraser University

Faculty of Health Sciences

Blusson Hall, Room 11300

8888 University Drive

Burnaby, B.C. V5A 1S6

Canada

Phone: 778-782-8494

Fax: 778-782-5927

Email: scott_venners@sfu.ca

5.

Perimenstrual Chocolate Cravings and the Physical and Affective Correlates of Menstruation

Julia M. Hormes, M.A.^{1,2}, Paul Rozin¹, Ph.D. & Ellen W. Freeman, Ph.D.³

¹Department of Psychology, University of Pennsylvania, 3720 Walnut Street, Philadelphia, PA 19104

²Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, 2500, N. State Street, Jackson, MS 39216

³Department of Obstetrics and Gynecology, University of Pennsylvania School of Medicine, 3701 Market Street, 8th Floor, Philadelphia, PA 19104

About 25% of American women crave chocolate perimenstrually, from several days before through the first few days of menstruation, and typically peaking on the day before its onset. Both estrogen and progesterone are at their lowest levels during the perimenstrum, and do not fluctuate substantially with the onset of menstruation. It was therefore hypothesized here that craving is a response to the subjective experience of stress during the perimenstrum, with the actual onset of menstruation yielding relief.

Participants were 164 women with premenstrual syndrome who completed the Daily Symptom Rating Scale (DSR) for two months of baseline ratings and one month of placebo administration. They rated 17 common premenstrual symptoms, loading on four factors of "Mood," "Behavior," "Pain" and "Physical," and the severity of their chocolate and other food cravings.

Marked increases in physical and affective symptoms as well as in craving severity occurred premenstrually. Only craving severity and ratings of affective symptoms, including depression and anxiety, decreased significantly with the onset of menses ($p \leq 0.05$), while physical symptoms and pain remained elevated.

Changes in craving severity and affective symptoms in the absence of fluctuations in hormones or physical symptoms speak against a causal role of hormones in the etiology of perimenstrual craving. Instead craving appears to be part of a cluster of psychological responses to the subjective experience of stress during the perimenstrual period. The onset of menstruation seems to exert its effect in decreasing some symptoms because of psychological (relief from anticipation) as opposed to hormonal/physical effects.

Julia M. Hormes

Email: jhormes@psych.upenn.edu

Phone: (601) 815-3688

Fax: (601) 984-5857

6.

Sentinels and Sensors: The DES-Exposed Human Offspring

Kari Christianson, Pat Cody, Karen Fernandes, Frances Howell,
Stephanie Kanarek, and Cheryl Roth

DES Action USA, P.O. Box 7296, Jupiter, FL 33468

For over 30 years DES Action USA has been the public voice for an estimated ten million individuals exposed to diethylstilbestrol (DES) in the United States alone. The focus of this non-profit consumer organization has been advocating for the exposed population. Advocacy includes encouraging research about human health outcomes, as well as recognizing important information found in studying endocrine disruption in the environmental and animal models. In the mid-1990s DES Action created a poster representing environmental questions about the effects of hormonally active substances that are capable of disrupting the endocrine systems of fish and wildlife, along with answers being sought about human health and reproduction in the DES-exposed population. Prenatal and generational DES exposures are both sentinel and central in studying and understanding the effects of endocrine disruption. The poster triptych is DES Action's first and only attempt to bring a visual representation about endocrine disruption on all life forms to the research community from the human population directly affected by DES.

Kari Christianson, Program Director, DES Action USA
kari@desaction.org
800-337-9288
612.383.5082 (cell)

7.

Exposure Assessment of Breastfed Infants to Persistent Organic Pollutants in Hungary

Éva Vigh¹, Andrea Colombo², Emilio Benfenati², Helen Håkansson³, Marika Berglund³,
József Bódis⁴, János Garai¹

¹Department of Pathophysiology and Gerontology; ⁴Department of Obstetrics and Gynecology; University of Pécs, Medical School, Pécs, HUNGARY

²Mario Negri Institute for Pharmacological Research, Department of Environmental Health Sciences, Milan, ITALY

³Karolinska Institute, Institute of Environmental Medicine, Stockholm, SWEDEN

Introduction: The manmade organochlorine compounds polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are persistent organic pollutants (POPs) that enter human body and accumulate in fatty tissues. These get easily transferred to breast milk hence breastfed infants might possibly be exposed to large amounts of potentially harmful POPs at the beginning of their life. This concern resulted world wide in breast milk POP studies, however, there is still insufficient data in Hungary.

Methods: 37 mothers delivered at Baranya County Teaching Hospital, Pécs, Hungary were recruited. Daily breast milk consumption of exclusively breastfed infants was recorded at regular periods during the first 3 months of lactation. Breast milk samples were collected from 34 mothers at 3 time points (week 1, 2 and 12) of the early lactation period. Milk samples were analyzed for PCBs, PCDD/Fs by HR GC-MS.

Results: The highest milk consumption per kg body weight was recorded at the end of the first month. Fat content remained stable during lactation. A declining trend was observed in milk PCB and PCDD/F congener concentrations. Concerning the whole first three months of the lactation period the infants' POP exposure was 5-6 times higher than the tolerable daily intake established by WHO.

Conclusion: Although the levels of pollutants in Hungarian breast milk samples are at the lower range in Europe, the exposure of breastfed infants to POPs exceeds tolerable daily intake proposed by WHO.

Presenting author: János Garai, E-mail: janos.garai@aok.pte.hu

Phone: +36 70 2483381

Fax: +36 72 536246

8.

Phytoestrogens and Other Plant Phenols Affect Macrophage Migration Inhibitory Factor Enzymatic Activity and Tubulin Polymerization

Éva Vigh, Piroska .Gabrieli, Edit Schumacher, Valéria Molnar, János Garai

Department of Pathophysiology and Gerontology, University of Pécs, Medical School,
Pécs HUNGARY

The rearrangement of microtubules occurs in conjunction with vesicle secretion upon hormone release, as well as with cell migration, phagocytosis and platelet aggregation that are common events in inflammation. The microtubule-associated proteins (MAP) determine the polymerization- depolymerization balance of tubulin units *in vivo*. An emerging body of evidence has linked recently macrophage migration inhibitory factor (MIF) with estrogen dependent inflammatory pathologies like osteoporosis, endometriosis and wound healing, however, its exact mechanism of action remains to be delineated. Our preliminary results have raised the possibility that the intracellular MIF cytokine might function as a MAP.

Enzymatic tautomerase and thiol-protein oxidoreductase activities of MIF have been described [1]. In our *in vitro* experiments the effect of plant polyphenols - including phytoestrogens – that are known to have anti-inflammatory effect were tested on MIF mediated tautomerism of phenylpyruvate, as well as on tubulin polymerization and on its phenylpyruvate tautomerase activity.

The plant polyphenols tested have, in a concentration-dependent way, inhibited not only the MIF mediated phenylpyruvate enol<>keto conversion but also the tautomerase enzymatic activity found in our tubulin preparations. The most effective inhibitors were caffeic acid, the phytoestrogen resveratrol, umbelliferon, phloretin, daidzein, naringenin and morin (IC₅₀-s in μ M for ketonase: 0.55, 1.91, 2.64, 4.81, 16.33, 29.52, 32.6 respectively). *In vitro* tubulin polymerization has also been inhibited by these polyphenols. These effects might be mediated through the enzymatic action of the microtubule associated MIF.

1. Rosengren E, et al: The macrophage migration inhibitory factor MIF is a phenylpyruvate tautomerase. FEBS Lett. 417: 85-88 (1997)

Presenting author: János Garai, E-mail: janos.garai@aok.pte.hu

Phone: +36 70 2483381

Fax: +36 72 536246

9.

Application of a Zebrafish Estrogen Receptor Binding Assay for Ecological Risk Assessment of Endocrine Active Substances

A. Tarrant¹, C. Mori², S. Thakali², M. Sharma², H. Yekel³ and T. Verslycke²

1. Woods Hole Oceanographic Institution, Woods Hole, MA, USA

2. Gradient Corporation, Cambridge, MA, USA

3. Wyeth Pharmaceuticals, Malvern, PA, USA

Fish and other aquatic species are exposed to diverse environmental pollutants, many of which have the potential to disrupt endocrine signaling. While the binding of natural and synthetic estrogens to mammalian estrogen receptors is well-described, the relative affinities of these compounds for estrogen receptors in other taxa are often unknown. We developed an *in vitro* fish estrogen receptor (ER) competitive binding assay to quantitatively assess the binding affinity of several synthetic estrogens used in drug formulations (e.g., estriol, 17 α -ethinylestradiol (EE2)). We expressed zebrafish ER- β 2 (zfER- β 2) *in vitro* with varying amounts of each estrogen along with tritiated estradiol and measured competitive displacement of tritiated estradiol from the receptor. The relative potency obtained from the *in vitro* assay was used to derive a predicted no (adverse) effect concentration (PNEC) for each estrogen, based on their relative binding affinities to zfER- β 2 and the literature-reported PNEC for EE2. These results were compared with receptor binding affinities obtained using other species and receptor forms. We propose that, along with the results from 'traditional' toxicity testing, this approach can be used to evaluate potential ecological risks of endocrine active chemicals. The proposed approach could also facilitate interpretation of *in vitro* screening results obtained through US Environmental Protection Agency's proposed Endocrine Disrupter Screening Program.

Ann Tarrant: Woods Hole Oceanographic Institution, 45 Water Street, Mailstop 33, Woods Hole MA 02543 USA. Phone 508 289 3398, Fax 508 457 2134, email atarrant@whoi.edu

10.

Localization of Steroid Receptors in the Chicken Yolk-sac Membrane

Pat Kasinpila, Lori C. Albergotti, and Louis J. Guillette, Jr.

Department of Biology, University of Florida, Gainesville, FL

Reptiles, birds, and mammals (amniotes) are defined by the formation of four extraembryonic membranes (the amnion, allantois, chorion, and yolk-sac), which play vital roles in embryonic development and survival. Of these, the yolk-sac membrane (YSM) functions in the uptake and modification of yolk lipids, thus providing the embryo with the nutrients required for growth and development in oviparous (egg-laying) species.

Numerous studies have revealed that the yolk of oviparous amniotes contains maternally deposited steroid hormones such as progestins, estrogens, androgens, and glucocorticoids. However, no investigations have examined if the YSM could receive and modulate maternally deposited yolk steroid hormones through the action of steroid receptors in the YSM. To begin investigating this question, we asked whether the YSM is capable of receiving progesterone and estradiol signaling through the appropriate steroid receptors. We examined protein localization of estrogen receptor alpha (ER α) and progesterone receptor (PRab) in the YSM of domestic chicken (*Gallus gallus*) using immunohistochemistry. We found that both ER α and PRab are present in the YSM indicating that the YSM is capable of receiving maternally deposited estradiol and progesterone during the uptake of yolk lipids. Our results suggest that the endocrine properties of extraembryonic membranes may not be limited to the placentae of viviparous (live-bearing) amniotes. Rather it appears that the extraembryonic membranes of oviparous amniotes are targets of endocrine signaling and we hypothesize that they also exhibit hormone synthesis or biotransformation abilities. At present, we are investigating whether the expression of ER α and PRab varies in the YSM during embryonic development.

Pat Kasinpila

Email: Patpilai@ufl.edu

Phone: 954-304-0041

Fax: 352-392-3704

11.

Estrogen Receptor α is Indispensable for the Induction of Persistent Vaginal Change by Neonatal 5 α -Dihydrotestosterone Exposure

Shinichi Miyagawa¹, Yoshinao Katsu¹, Yasuhiko Ohta², Tamotsu Sudo³, Dennis B. Lubahn⁴
and Taisen Iguchi^{1,*}

¹Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, and Department of Basic Biology, The Graduate School for Advanced Studies, Okazaki 444-8787, Japan; ²Department of Veterinary Medicine, Faculty of Agriculture, Tottori University; ³Section of Translational Research and Development of Gynecological Oncology, Hyogo Cancer Center, Akashi 673-8558; ⁴Department of Molecular Biology, University of Missouri, Columbia, Missouri 65211, USA.

Development of the reproductive organs can be strongly affected by the hormonal environment. In the mouse, exposure to sex steroid hormones during the critical period induces estrogen-independent cell proliferation and differentiation in the vaginal epithelium, which often results in cancerous lesions later in life. In the present study, we assessed the contributions of estrogen receptor α (ER α) to the developmental effects of 5 α -dihydrotestosterone (DHT) on female mouse vagina and external genitalia. The vagina of ER α ^{-/-} mice treated neonatally with DHT showed atrophic epithelium, whereas the vaginal epithelium of ER α ^{+/+} mice was stratified and keratinized even after ovariectomy. In addition, neonatal treatment with DHT led to persistent phosphorylation of ER α in the vaginae of 60-day-old, ovariectomized mice. We infer from these data that ER α is obligatory for the induction and maintenance of persistent vaginal epithelial changes induced by neonatal administration of DHT. In contrast, neonatal DHT treatment induced hypospadias in both ER α ^{-/-} and ER α ^{+/+} mice. We found that os penis-like large bone formation in the clitoris was induced in ER α ^{-/-} mice, but not in ER α ^{+/-} or in ER α ^{+/+} mice, indicating the hyper-responsiveness of ER α ^{-/-} mouse external genitalia to DHT. The current results shed light on the mechanisms of induction of developmental effects elicited by sex steroid hormones on the developing animals.

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Taisen Iguchi: taisen@nibb.ac.jp, phone: +81-564-59-5235, fax: +81-564-59-5236

12.

Rapid Regulation of K_{ATP} Channel Activity by 17 β - Estradiol in Pancreatic β -Cells Involves the Estrogen Receptor β and the Atrial Natriuretic Peptide Receptor

Sergi Soriano¹, Ana B. Ropero¹, Paloma Alonso-Magdalena¹, Cristina Ripoll¹, Ivan Quesada¹, Esther Fuentes¹, Brigit Gassner², Michaela Kuhn², Jan-Ake Gustafsson^{3,4} and Angel Nadal¹

¹Instituto Bioingeniería and CIBERDEM, Universidad Miguel Hernández, Elche 03202, Alicante, Spain. ²Institute of Physiology, University of Würzburg; Germany. ³Department of Biosciences and Nutrition, Karolinska Institute, Novum, Huddinge, Sweden. ⁴Department of Cell Biology and Biochemistry, Center for Nuclear Receptors and Cell Signaling, University of Houston, USA

The ATP-dependent potassium channel (K_{ATP} channel) is a key molecule involved in glucose-stimulated insulin secretion (GSIS). The activity of this channel regulates β -cell membrane potential, glucose-induced $[Ca^{2+}]_i$ signals and insulin release. In this study, the rapid effect of physiological concentrations of 17 β -estradiol (E2) on K_{ATP} channel activity was studied in intact β -cells using the patch-clamp technique. When cells obtained from wildtype (WT) mice were used, 1nM E2 rapidly reduced K_{ATP} channel activity by 60%. The action of E2 on K_{ATP} channel was not modified in β -cells from ER α -/- mice yet it was significantly reduced in cells from ER β -/- mice. The effect of E2 was mimicked by the ER β agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), while the ER α agonist propylpyrazole-triol (PPT) was inactive. In addition, activation of ER β by DPN enhanced glucose-induced Ca^{2+} signals and insulin release in freshly isolated murine islets of Langerhans. Previous evidence indicated that the acute inhibitory effects of E2 on K_{ATP} channel activity involve cyclic GMP (cGMP) and cGMP-dependent Protein Kinase (PKG). In this study, we used β -cells from mice with genetic ablation of the membrane guanylate cyclase A (GC-A) receptor for ANP (also named: atrial natriuretic peptide receptor) (GC-A KO mice) to demonstrate the involvement of this membrane receptor in the rapid E2 actions triggered in β -cells. E2 rapidly inhibited K_{ATP} channel activity and enhanced insulin release in islets from WT mice but not in islets from GC-A KO mice. Additionally, DPN reduced K_{ATP} channel activity in β -cells from WT mice but not in those from GC-A KO. This work unveils a new role for ER β as an insulinotropic molecule that may have important physiological and pharmacological implications.

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A. Nadal, phone: 34 965222002; fax 34 965222033; e-mail: nadal@umh.es

E. Fuentes, phone: 34 965222010; fax 34 965222033; e-mail: efuentes@umh.es

13.

Cadmium and Other Metals Ions Lack Endocrine Disrupting Activity in Yeast and Mammalian Reporter Assays

Xiaobing Tan¹, Thomas Wiese² and Charles Miller¹

Department of Environmental Health Sciences, Tulane University School of Public Health and Tropical Medicine¹ and the Xavier University of Louisiana, College of Pharmacy²,
New Orleans, LA

Previous reports suggest that cadmium and other metal ions may act directly or indirectly to alter steroid hormone receptor signaling. Here, recombinant yeast engineered to express human androgen, estrogen α or β , glucocorticoid, mineralocorticoid, or progesterone receptors and relevant lacZ reporter genes were used to survey Cd⁺² and other metal ions (iron, mercury, lead, copper, arsenic) for endocrine disrupting activity. No agonist activity for any of the six steroid hormone receptors was detected across broad ranges of metal concentrations. Each receptor-bearing yeast strain was then activated with cognate steroid hormone in a second series of experiments and a range of metal concentrations was used to assess signaling inhibition. Metal ions caused signaling inhibition, but only at concentrations that were near or within the range that also retarded growth or killed cells. Thus, inhibition of steroid hormone receptor signaling by metal ions probably resulted from general rather than selective toxicity of the metal ions. Assessment of metal ion-induced activation of estrogen receptor signaling was conducted in recombinant breast cancer cell lines (MVLN and T47D-KBluc) with different ERE-directed luciferase reporter genes. As observed in the yeast system, no evidence for estrogenic activity of any of these metal salts was found. Our results clearly show that metal ions (e.g. Cd⁺²) are not simple activators of steroid hormone receptor signaling, and are only inhibitory at generally toxic doses.

Charles A. Miller III, Ph.D.
Professor of Environmental Health Sciences
Tulane University School of Public Health and Tropical Medicine
Suite 2100, Tidewater Bldg.
1440 Canal Street
New Orleans, LA 70112

504-988-9061, rellim@tulane.edu

14.

Toxic and Genotoxic Studies of Wood Dusts

Wilson, Mark 1; Rando, Roy 1, 4; Miller, Charles1, 2, 3; Tan, Xiaobing1

1. Environmental Health Sciences, Tulane University, New Orleans, LA, USA.
2. Tulane Cancer Center, Tulane University, New Orleans, LA, USA.
3. Center for Bioenvironmental Research, Tulane University, New Orleans, LA, USA.
4. Environmental Medicine, Tulane University, New Orleans, LA, USA.

The National Toxicology Program and the International Agency for Research on Cancer have generally classified wood dust as a known human carcinogen. This classification is based on epidemiologic studies, which clearly linked an increased risk of nasal adenocarcinoma with chronic occupational exposures to wood dusts. However, few reports have addressed which wood dusts are of the greatest concern, and the potential mechanisms of action of wood dust induced toxicity are relatively unexplored. To address some of these informational gaps we used a panel of in vitro assays to investigate the toxic and genotoxic potential of dusts from different woods and wood products types. Our studies indicated that dusts from specific woods and wood products contained a soluble compound(s) that 1) potently activated aryl hydrocarbon receptor signaling, 2) generated changes in mammalian cell ploidy, and 3) induced mutations in a target transgenic gpt gene in mammalian cells. Additional studies to identify the mutation spectra of wood dusts are ongoing. We conclude that some wood dusts are particularly genotoxic and thus may pose potentially greater health risks than others. Teak wood dust may be of particular concern to woodworkers because it always displayed the most activity in these in vitro assays.

Contact information

Mark Wilson

Mwilson9@tulane.edu

504-988-6525 fax 504-988-1726

15.

Alkylphenols Affect Multiple Responses in Lobster Molting and Metamorphosis

Hans Laufer^{1,5}, Ming Chen¹, Brian Baclaski¹, James Stuart², James Bobbitt², Molly W. Jacobs^{1,4}, Michael Johnson¹, Yuegang.Zuo³, Zhuo Zhu.³

1. University of Connecticut, Department of Molecular and Cell Biology 2. U.Conn, Dept. of Chemistry 3. U.Mass, Dartmouth 4. Woods Hole Oceanographic Inst. 5. Marine Biological Laboratory, Woods Hole, MA.

Alkylphenols are derived from the manufacture and use of plastics, detergents, and antioxidants. The aquatic environment is contaminated from their production, use and disposal. Scientists are concerned because of their toxicity and estrogenic endocrine disrupting activities. As much as 50% of lobsters in New England were contaminated with at least 1, or up to 6 compounds in their blood or tissues, at concentration of 1 to 2 µg/gm wet weight. The compounds are 2-t-butyl-4-(dimethylbenzyl) phenol (Comp. 1), 2,6-bis-(t-butyl)-4-(dimethylbenzyl) phenol (Comp. 2), 2,4-bis-(dimethylbenzyl) phenol (Comp. 3), and 2,4-bis (t-butyl)-4-(dimethylbenzyl)-6-butylphenol (Comp. 4), 4-cumylphenol and bisphenol A (BPA). In vitro incorporation of C¹⁴-tyrosine and C¹⁴-Comp. 3 into molting cuticle was dependent on phenoloxidase. Unlabeled Comp. 3 competed more effectively than tyrosine, or its derivatives, which normally crosslink proteins, and function in shell hardening, weakening lobster shells, possibly making them more susceptible to microbial invasion.

Administration of Comp. 3 or BPA to larvae (5 or 10 ng/ per day/larva) in food, resulted in increased mortality (69% controls survived vs. 13-14% and 16-21% for Comp. 3 and BPA, respectively). Methylfarnesoate (MF) treatment, which has juvenile hormone activity in lobsters, resulted in larval intermediates at metamorphosis. Untreated controls metamorphosed into juveniles, while treated survivors (n=35) metamorphosed into intermediates (n=22 or 40-88% depending on treatment) by hormone disruption. Molting was delayed by three to four days in treated larvae.

In conclusion, alkylphenols are toxic estrogenic endocrine disruptors interfering in lobster survival, molting, and metamorphosis. They have multiple mechanisms of action, adversely affecting lobster health.

Contact Information:

Hans Laufer, Research Professor
Department of Molecular and Cell Biology
University of Connecticut
91 North Eagleville Rd. U-3125
Storrs, CT 06269-3125
Phone: (860) 486-4117
Fax: (860) 486-4331
Laufer@uconn.edu

16.

Differences Between Rat and Human Metabolism of Bisphenol A: Using *In Vitro* Kinetic Parameters To Extrapolate To *In Vivo* Intestinal Metabolism Rates

Janet K. Hess-Wilson¹, Christopher S. Mazur², John F. Kenneke², John C. Lipscomb¹

¹ U.S. Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment, Cincinnati, Ohio

² U.S. Environmental Protection Agency, Office of Research and Development, National Exposure Research Laboratory, Athens, Georgia

Bisphenol A (BPA) is widely used in the production of polycarbonate plastics and epoxy resins, classified as an endocrine disruptor, and has reported effects on the reproductive organs of animals. The primary route of human exposure to BPA is via oral exposure. Following ingestion in both rats and humans, BPA metabolism occurs via phase II conjugation to BPA-monoglucuronide (BPAG). BPAG is not estrogenic; therefore, metabolism is the essential mechanism for limiting systemic bioavailability and estrogenic effects. There are noted species and gender differences in BPA body burden, the reasons of which are unknown. The pharmacokinetics of BPA are complicated by the interspecies differences in the involvement of the gastrointestinal tract in glucuronidation. Using liver and intestinal microsomes, we determined BPA metabolism parameters for rats and humans. Male- and female-specific liver microsomes were also assessed to determine the extent of sex-specific metabolism. Our *in vitro* results determined that the V_{max} for intestinal metabolism of BPA is almost 30 times higher in rats compared to humans (18,795 and 660 pmoles/min/mg, respectively). We then used scaling factors to extrapolate the kinetic data from *in vitro* systems to *in vivo* parameters. We elucidate the importance of species-specific parameters for intestinal metabolism of BPA and provide *in vivo* extrapolated values for incorporation into physiologically-based pharmacokinetic (PBPK) models. Our data will help reduce uncertainty in quantifying metabolic information for rodents and humans and improve the utility of PBPK models. Therein, more accurate tissue dosimetry can be determined to assess the impact of BPA on human health.

26 W. Martin Luther King Dr.
Cincinnati, OH 45268
hess-wilson.janet@epa.gov
(p) 513-569-7458
(f) 513-487-2540

17.

Tissue Reconstruction: Uterine Fibroids and the Possible Role of Bisphenol A

Chasity B. Coleman¹ and John A. McLachlan^{1,2,3}

¹Center for Bioenvironmental Research, Tulane University, New Orleans, Louisiana;

²Tulane Cancer Center, Tulane University, New Orleans, Louisiana; ³Department of Pharmacology, Tulane Medical School, New Orleans, Louisiana

Uterine fibroids are benign smooth muscle tumors that affect nearly 13.6 million women in the United States, resulting in 150,000-175,000 hysterectomies each year, but have no known cause. Studies from our laboratory and others have shown that there are differences in cell signaling and gene expression between fibroids and adjacent myometrial cells, with fibroid cells missing a “brake” in signaling, allowing estrogen to trigger an unregulated feedback loop leading to changes in cell growth and gene expression. The link between these differences in cells in tissue culture and in vivo fibroid formation is not clear. In the environment, endocrine disrupting compounds, such as bisphenol A, exhibit estrogenic effects and have been linked to fibroids. To date there have been no experiments published examining the direct effects of bisphenol A on uterine leiomyoma in cell culture, leaving a major gap in both bisphenol A and leiomyoma research. Our study has two aims: to investigate the role of bisphenol A in growth of uterine fibroids and to explore novel methods for growth of fibroids in culture. When exposed to bisphenol A in traditional cell culture, uterine fibroid cells increased proliferation in a dose dependent manner, but normal smooth muscle cells did not exhibit the same dose dependence. These results led us to investigate three-dimensional cell culture for growing uterine fibroid cells in a more true to life manner. We are now perfecting techniques for growing fibroid and normal smooth muscle cells in three-dimensional cell culture to use in future experiments.

Chasity B. Coleman, PhD, ccoleman@tulane.edu, (504) 988-6623, fax: (504) 988-6215

18.

Isolation Of Tumor Initiating Cells With Metastatic Potential From Human Primary Invasive Ductal Carcinoma

Carolyn Marsden^{*†}, Mary Jo Wright^{††}, Latonya M. Carrier^{*†}, Radhika Pochampally^{•†▲} and Brian G. Rowan^{*†}

Departments of Structural and Cellular Biology^{*}, Surgery[†], Pharmacology[•], Environmental Health Sciences^{**}, and Tulane Cancer Center[†], and Center for Gene Therapy[▲], Tulane University Health Science Center, New Orleans LA 70112.

Disseminated breast cancer cells may be present at distant sites at the time of primary diagnosis of breast cancer in patients that exhibit no outward signs of clinical metastasis. It is hypothesized that early breast cancer metastasis is initiated by a small population of breast tumor initiating cells (bTICs) within the primary tumor that are inherently resistant to chemotherapy and hormonal therapy. To better understand the biological pathways that permit bTICs to metastasize and to evade current breast cancer therapies, bTICs have been isolated from breast cancer biopsies from patients diagnosed with primary invasive ductal carcinoma. Breast cancer needle biopsies from primary tumors were obtained during the routine care of patients with consent and IRB approval. Core biopsies were mechanically and enzymatically dissociated, yielding a single cell suspension which was subsequently cultured under non-adherent and serum-free conditions to obtain tumorspheres. Injection of about 500 bTICs into the mammary fat pad of female nude mice resulted in tumor formation at the site of injection within two months that was maintained as a small palpable mass for at least six months. H+E staining of sections of the primary tumors revealed complex cellular organization and both microvasculature and macrovasculature. Serial transplantation of the primary tumors from 6/7 samples resulted in tumor formation at the site of injection. Upon primary tumor formation in the mammary fat pad, metastatic human breast cancer cells were detected within the lungs, liver, brain and bone marrow (femur) from 5/7 tumors after six months. Metastatic cells were detected by PCR for an alpha-satellite sequence in the centromeric region in human Chromosome 17, and by in situ hybridization using a human specific Alu DNA oligonucleotide probe. Metastatic bTICs were mostly detected as single cells or small clustering of cells present throughout the metastatic organ. These data demonstrate that cells with the phenotype of "tumor initiating cells" can be isolated from primary biopsies of human invasive ductal carcinoma and cultured *in vitro*. Unlike direct heterotransplant tissues from primary tumor biopsies, the majority of bTICs form tumors when injected into immunodeficient mice and further exhibit a highly metastatic phenotype.

Carolyn Marsden

cmarsden@tulane.edu

(504) 988-1562-phone

(504) 988-1687-fax

19.

CXCR4 Expression Mediates Hormone Independence and Endocrine Therapy Resistance through Erk1/2 and p38 Signaling

Lyndsay V. Rhodes¹, Nicole F. Neel³, Yun Zhu¹, Virgilio A. Salvo¹, Juan P. Fonseca¹, Steven Elliott¹, Henry C. Segar¹, Becky Worthylake⁴, Bridgette Collins-Burow¹, Mien-Chie Hung⁵, Ann Richmond^{2,3}, Matthew E. Burow¹

¹Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana, Departments of ²Veterans Affairs and ³Cancer Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, ⁴Department of Pharmacology, Louisiana State University Health Science Center, New Orleans, Louisiana, ⁵Department of Molecular and Cellular Oncology, University of Texas M. D. Anderson Cancer Center, Houston, TX

Chemokine X Receptor 4 (CXCR4) expression is critical to cancer cell invasion and metastasis. Altered expression and activation by its ligand, stromal-derived growth factor 1 (SDF-1), mediates breast carcinoma progression to a more metastatic phenotype. The association of a metastatic phenotype with hormone independence, along with SDF-1 being an estrogen receptor (ER) regulated gene, establishes a link between hormone and chemokine signaling in breast carcinoma. SDF-1-CXCR4 signaling promotes proliferation, cell motility/invasion, and suppresses apoptosis through activation of signaling pathways including mitogen-activated protein kinase (MAPK). Interestingly, MAPKs have been implicated in endocrine therapy resistance. While endocrine therapy holds great promise in treatment of hormone-dependent cancer, many patients display resistance, either acquired or *de novo*. Resistance primarily occurs through altered signaling cascades leading to ligand-independent activation of estrogen receptor mediated gene expression and hormone independence.

As reported here, a deletion in the COOH-terminal domain confers constitutive activity of CXCR4 leading to enhanced cell growth and metastases *in vivo*. Furthermore, overexpression of CXCR4 in MCF-7 leads to hormone independence *in vivo*. Treatment with exogenous SDF-1 negates inhibitory effects of anti-estrogen treatment on MCF-7-CXCR4 tumor growth indicating involvement in endocrine therapy resistance. These effects are correlated with CXCR4 mediated activation of downstream signaling events (Erk1/2, p38) and enhancement of ER-mediated gene expression. These results indicate that, in addition to mediating metastatic potential of ER + breast carcinoma, CXCR4 signaling contributes to hormone independence and endocrine therapy resistance through Erk1/2 and p38 signaling. Better mechanistic understanding of hormone independence/endocrine therapy resistance is paramount to discovery and utilization of novel treatment targets.

Lyndsay V. Rhodes; lvanhoy@tulane.edu; (504) 988-6704

20.

Organochlorine-mediated Phosphorylation of the General Coactivator CBP through p38 Mitogen Activated Protein Kinase

Melyssa R. Bratton^{§§¶ Δ} , Daniel E. Frigo^{¶§¶ Δ} , Katinka A. Vigh[¶], Daju Fan^{||}, Scott Wadsworth[○], John A. McLachlan^{¶§§}, and Matthew E. Burow^{¶**##¥}

§Molecular and Cellular Biology Program, ¶Center for Bioenvironmental Research, **Department of Medicine-Section of Hematology and Medical Oncology, ##Department of Surgery, and the §§Department of Pharmacology, Tulane University Health Science Center, New Orleans, LA 70112, ||Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710, and ○Johnson & Johnson Pharmaceutical Research & Development, Raritan, NJ 08869-0602.
email: mbratton@tulane.edu, ph: 504-899-2963

The activity of nuclear transcription factors is often regulated by specific kinase-signaling pathways. We have previously shown that the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT) stimulates activator protein-1 activity through the p38 mitogen-activated protein kinase (MAPK). We have also shown that DDT and its metabolites stimulate the transcriptional activity of cyclic adenosine monophosphate response element-binding protein (CREB) and Elk1 and potentiate gene expression through cyclic adenosine monophosphate and hypoxia response elements. Because DDT stimulates gene expression through various transcription factors and hence multiple response elements, we hypothesized that p38 signaling may target a common shared transcriptional activator. Here, we demonstrate using both pharmacological and molecular techniques, the general coactivator CREB Binding Protein (CBP) is phosphorylated and potentiated by the p38 MAPK signaling cascade. We further show that p38 directly phosphorylates CBP in its C-terminus. These results, together with our previous work, suggest that the organochlorine DDT stimulates p38 MAPK which subsequently phosphorylates and activates downstream transcription factors in part by targeting the general coactivator CBP.

21.

Overexpression of miR-155 Disrupts ER Signaling in MCF-7 Breast Cancer Cells

Elizabeth Martin¹, Syreeta Tilghman⁴, Steven Elliott¹, Mona Jodari-Karimi¹, Lyndsay Rhodes¹, Erik Flemington³, and Matthew E. Burow^{1,2,5}

Departments of ¹Medicine, ²Pharmacology, ³Pathology and Laboratory Medicine, ⁴Pulmonary Diseases Critical Care and Environmental Medicine and ⁵The Center for Bioenvironmental Research, Tulane University, New Orleans, LA

microRNAs (miRNA) are short non-coding RNAs that regulate gene function through translational inhibition. Changes in miRNA expression patterns have been linked to diseases such as cancer and differences in miRNA expression profiles have been shown to correlate with breast cancer receptor status, including receptors ER, PgR, and HER2/Neu. Here we demonstrate ER regulation of several miRNAs after treatment with 17beta estradiol, 4-OH-tamoxifen, and ICI 182,780 in the MCF-7 breast cancer cell line. Specifically we show that miR-155 expression is altered after treatment with 17beta estradiol. Furthermore ectopic expression of miR-155 in MCF-7 breast cancer cells results in a dysregulation in the ER signaling pathways both *in vivo* and *in vitro*. Notably overexpression of miR-155 leads to changes in the expression patterns of ER regulated genes PGR and BCL2 as well as, a suppression of estrogen-stimulated clonogenicity and tumorigenesis. Taken together our data suggests a role for miR-155 in the regulation of ER signaling and breast tumorigenesis.

22.

Endocrine Disruptor Regulation of miR-21 Expression in Breast Cancer Cells

Syreeta L. Tilghman¹, Melyssa R. Bratton², Henry C. Segar, Elizabeth Martin, Hideo Iba³, John A. McLachlan^{2,4}, Thomas Wiese⁵, Kenneth Nephew⁶ and Matthew E. Burow^{1,4}

School of Medicine, Section of Hematology & Medical Oncology¹, Pharmacology², Tulane University, New Orleans, LA 70112. Institute of Medical Science³, University of Tokyo, Tokyo, Japan. Center for Bioenvironmental Research⁴, Tulane University, New Orleans, LA 70112, College of Pharmacy⁵, Xavier University, New Orleans, LA 70125. School of Medicine⁶, Indiana University, Bloomington, Indiana 47405

Endocrine disrupting compounds (EDCs) such as the organochlorine pesticide, dichlorodiphenyltrichloroethane (DDT), and the plasticizing agent, bisphenol A (BPA), represent established and emerging environmental estrogenic agents of potential concern that have a great impact on human health. Recently, the field of microRNAs has emerged. A number of studies have demonstrated that specific microRNAs target and deregulate a several genes involved in breast cancer. Previous reports have shown estradiol to target and downregulate miR-21 expression and increase miR-21 target gene expression in MCF-7 breast cancer cells. However no studies have been performed to investigate the effect of EDCs on miR-21 expression in breast cancer. Therefore, in this study we tested the hypothesis that miR-21, is regulated by specific EDCs in MCF-7 breast cancer cells. Here, we report that both BPA and DDT treatment are estrogenic as they increase the estrogen response element (ERE) transcriptional activity in MCF-7 cells. We used a focused PCR array approach to examine regulation of key estrogen receptor (ER) responsive genes and found like E₂, BPA and DDT induced the gene expression of progesterone receptor (PgR), pS2 and cathepsin D. A microRNA microarray was performed on MCF-7 cells exposed to E₂, BPA and DDT and results revealed a downregulation in miR-21 expression. The BPA and DDT-mediated decrease in miR-21 correlated with increased gene expression of endogenous miR-21-targets PDCD4 and maspin. Taken together, these results are the first to demonstrate that BPA and DDT repress the expression of an oncogenic miRNA, miR-21.

23.

Sphingosine Kinase: A Novel Therapeutic Target for ER Signaling in Breast Cancer

James W. Antoon¹, Martin D. White¹, William D. Meacham¹, Evelyn M. Slaughter¹, Shannon E. Muir¹, Steven Elliott², Lyndsay V. Rhodes², Hasina B. Ashe³, Thomas E. Wiese³, Charles D. Smith⁴, Matthew E. Burow², Barbara S. Beckman¹

Tulane Departments of Pharmacology¹, Medicine, Section of Hematology & Medical Oncology², Tulane University School of Medicine, New Orleans, LA 70112; College of Pharmacy³, Xavier University, New Orleans, LA 70112; Department of Pharmaceutical and Biomedical Sciences⁴, Medical University of South Carolina, Charleston, SC 29425.

Alterations in sphingolipid metabolism have been shown to contribute to the development of endocrine resistance and breast cancer tumor survival. Sphingosine kinase (SK), in particular, is overexpressed in breast cancer and has been proposed as a major target for breast cancer drug development. We investigated the ability of two sphingosine kinase inhibitors SKI-II (4-[4-(4-chloro-phenyl)-thiazol-2-ylamino]-phenol) and ABC294640 (3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl) amide) to target estrogen receptor- positive signaling in breast cancer cells. We found that SKI-II and ABC294640 were effective in diminishing MCF-7 clonogenic survival, with IC₅₀ values of 2.04µM and 3.15µM, respectively. Reporter gene assays revealed that these drugs decrease estrogen-stimulated ERE-luciferase activity in both MCF-7 and ER-transfected HEK293 cells. Furthermore, these sphingosine kinase inhibitors blocked estrogen-target gene transcription of downstream ER Signaling. Treatment with SKI-II and ABC294640 reduced transcription of the known ER mediated genes progesterone receptor, and SDF-1. Here, we propose that these inhibitors block ER signaling through direct binding with the estrogen receptor. Enzymatic binding assays show SKI-II and ABC294640 can bind the antagonist ligand binding domain of the ER, acting as partial antagonist in a similar method to tamoxifen. Finally, treatment with ABC294640 effectively diminished breast cancer tumor formation *in vivo*. After 14 days of treatment with ABC294640, tumor volume was reduced by 67% (n=5), with no marked weight loss or illness. Taken together, these results provide strong evidence that selective sphingosine kinase inhibitors have therapeutic potential in treating ER-positive breast cancer via inhibition of signaling between sphingosine kinase and ER-regulated gene transcription.

Presenting Author Contact Information: jantoon@tulane.edu.

504-988-5589, 504-988-5283

24.

KX-01, a Novel Src kinase Inhibitor directed towards the Peptide Substrate site, Induces Robust Apoptosis and Synergizes with Tamoxifen and Chemotherapy in Breast Cancer

Muralidharan Anbalagan^{*}, Latonya Carrier^{*}, David Hangauer^{**},^{***} and Brian G. Rowan^{*}

^{*}Department of Structural and Cellular Biology, Tulane University School of Medicine, New Orleans, LA, ^{**}Kinex Pharmaceuticals LLC, ^{***}State University of New York at Buffalo, Buffalo, New York

New therapeutic regimens that increase efficacy or reduce onset of resistance for endocrine therapy and chemotherapy are needed for better clinical management of breast cancer. c-Src is an oncogenic non-receptor tyrosine kinase that is up-regulated in approximately half of all breast cancer. However the efficacy of existing multi-kinase src inhibitors in breast cancer has been limited. KX-01 (Kinex Pharmaceuticals) is a novel class of non-ATP src inhibitor that targets the peptide binding site of src and is currently completing Phase-1 testing for solid tumors. In a panel of breast cancer cell lines, KX-01 resulted in dose dependent inhibition of growth and induction of apoptosis that was independent of p53 status, and was preceded by rapid inhibition of src activity. KX-01 induced apoptosis in two cell lines reported to be resistant to multi-kinase src inhibitors, MDA-MB-468 cells and BT-549. Cell cycle analysis revealed that KX-01 (50 nM, 6 hours) resulted in significant accumulation of MDA-MB-231 cells (ER α /PR/HER2/neu negative) and MCF-7 cells (ER α positive) in G2/M phase. Immunofluorescent staining for mitotic phase marker phospho-histone 3 indicated that cells had arrested in mitotic phase and many of the mitotic arrested cells were undergoing apoptosis (TUNEL), a novel cell death for a small molecule tyrosine kinase inhibitor. KX-01 induced nuclear accumulation of cyclin B1, and activation of CDK1, MPM2 and Cdc25C that is required for progression past the G2/M checkpoint. KX-01 resulted in cytochrome C release and activation of caspases 3, 6, 7, 8 and 9. A matrix design using the median-effect principle to delineate the interaction between two drugs was applied for KX-01 alone and in combination tamoxifen (TAM), paclitaxel (PAC) or doxorubicin (DOX). Combinations of KX-01 (5-75 nM) with each of these agents resulted in synergistic growth inhibition of MCF-7 cells (KX-01 + TAM) and MDA-MB-231 cells (KX-01 + DOX or PAC). In addition, synergistic induction of apoptosis was achieved by combining low doses of KX-01 with DOX, PAC or TAM. c-Src induces phosphorylation of ER α at serines 118 and 167, sites required for full receptor activity. KX-01 combined with TAM resulted in decreased phosphorylation at serine 167 that was associated with reduced transcriptional activity of ER α . In tumor xenograft models, KX-01 resulted in a dose dependent inhibition of MDA-MB-231 and MCF-7 tumor growth after 30 days (1, 2.5 or 5 mg/kg body weight, twice daily by oral gavage). In MDA-MB-231 xenografts, KX-01 reduced metastasis to bone (femur) and lung as measured by PCR for detection of human chromosome 17. These data define KX-01 as a potently active src kinase inhibitor that induces robust cell death, tumor growth inhibition and anti-metastatic effects. Combinations of KX-01 with endocrine therapy and chemotherapy present a promising new strategy for clinical management of breast cancer.

Presenting Author: Muralidharan Anbalagan

Email: manbalag@tulane.edu

Phone: 504-988-1563

Fax: 504-988-1687

25.

***In Vitro* Subcutaneous Absorption of Fenthion using Female Abdominal Skin**

Annette M. Hormann

Southern Illinois University-Carbondale, Illinois 62901 USA

Primary exposure routes for organophosphates are through inhalation, ingestion, and dermal absorption. When the neuromuscular system is compromised depending on the amount, it can impair or kill the agricultural worker. Organophosphates account for 33% of all pesticide related poisoning reported by the Environmental Protection Agency. Health outcomes from an exposure can result in reproductive harm, carcinogenic mutations, and endocrine disruption.

This pilot-study used fenthion, an organophosphate pesticide, with the primary route of exposure in the abdominal area of females. The research focused on the difference that could exist between the experimentally derived permeability-coefficients and the calculated permeability-coefficients from the literature. Previously performed experiments showed it was feasible to develop experimentally based estimates for permeability-coefficients of skin absorption and penetration.

Flux measurements were taken at five specific points in time during non-steady state and then compared to the predicted results using steady-state equations. Three different concentrations of Fenthion were tested using *in vitro* technology with skin samples analyzed on the gas chromatogram (EPA method 8141B). Absorption (flux) was measured and permeability-coefficients (P) were calculated based on Fick's law of diffusion. Experimentally derived permeability-coefficients were compared with predictive models. Electrical capacitance was used to determine if the skin was breached.

Results showed that there was not a significant difference from most of the models in the literature. This study supports the premise of developing scientifically-defensible experimentally based estimates for permeability-coefficients of dermal absorption and penetration. Furthermore, this suggests that non-steady state can be used in the predictive equations to calculate permeability.

Annette M. Hormann

AnnetteH@siu.edu

573-587-2303 cell

573-243-4876 fax

26.

Interactions of Estrogenic Pesticides on Breast Cancer Cell Gene Expression Evaluated with a Cancer Focused PCR Array System

H. Chris Segar^{1,2}, Elena V. Skripnikova², Huiming Li², Thomas E. Wiese²

¹Biomedical Sciences Program, Tulane University School of Medicine, ²Xavier University of Louisiana College of Pharmacy, New Orleans, LA

A number of pesticides have been shown to stimulate estrogen receptor (ER) mediated proliferation and induce gene expression in breast cancer cells in culture. Thus, exposure to such compounds could be a contributing factor in the progression of breast cancer. We have previously described the estrogen regulated proliferation and reporter gene activity of DDT isomers and metabolites as well as of methoxychlor and its primary metabolite HPTC. In addition, we have shown that some organophosphate pesticides may potentiate this estrogen activity in particular binary mixtures. The goal of this study is to characterize the interactive effects of binary mixtures of pesticides and metabolites with estrogen activity on breast cancer cell gene induction. Most published characterizations of the hormone activity of pesticides in breast cancer cells have measured only the effects of single, pure compounds. Considering that real life exposure includes multiple isomers and/or the production of metabolites, it is of interest to examine what effect a mixture of these isomers and metabolites may have on genes related to breast cancer etiology and progression. MCF-7 cells were treated with mixtures of estradiol and one of the test pesticides as well as various mixtures of two pesticides, including combinations of one DDT and one of the organophosphate pesticides fenitrothion, methylparathion or parathion. RNA collected from these cultures was then used in Breast Cancer and Estrogen Receptor Signaling PCR Arrays from SA Bioscience. Analysis of these arrays in relation to each other and arrays from controls (blank, estradiol, single pesticides) are presented with highlight of novel gene expression patterns resulting from binary pesticide mixtures.

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Chris Segar
hsegar@tulane.edu

27.

Sample Preparation of Wastewater and Dissolved Solids for Use in an Estrogen Responsive Cellular Bioassay

Adam B, Streiffer¹, Thomas Wiese², Ponsawat Srisawat³, Deborah Grimm⁴

¹ERM, 3838 N. Causeway Blvd., STE 2725, Metairie, LA 70002

²Xavier University of Louisiana College of Pharmacy, New Orleans, LA, 70125

³Dept. of Environmental Health, School of Public Health, Tulane University, New Orleans, LA, 70112

⁴Coordinated Instrumentation Facility, Tulane University, New Orleans, LA, 70118

Bioassay analysis of Endocrine Disrupting Chemicals (EDCs) in wastewaters allows for the determination of the full hormonal response of the sample, but presents unique sample preparation challenges due to the sensitivity of bioassays to experimental conditions. We developed a novel sample preparation method for liquid and solid phases of wastewaters for use in bioassay testing. Wastewater samples were analyzed using a MVLN bioassay, which quantitatively measures estrogen activity through the activation of the endogenous estrogen receptor. To demonstrate adequacy of the method, qualitative GC/MS analysis of a subset of samples was performed. Preliminary results indicate that this methodology is an effective technique for preparation of wastewater samples for hormonal analysis in cellular bioassays. This method has potential crossover applicability to other media including drinking water and sludges, as well as use in the development and testing of EDC treatment methods, and improved assessment of ecological and public health impacts of water discharges.

Correspondence should be addressed to: adam.streiffer@erm.com

28.

A Compilation of Suspected Environmental ED Sites in Louisiana

Rhea Vidrine, Gary LaFleur, Jr.

Biological Sciences, Nicholls State University, Thibodaux, LA 70310

gary.lafleur@nicholls.edu

The endocrine system is comprised of several glands that regulate the body's metabolic activity through hormone secretions. Chemicals that have the ability to disrupt the hormone signaling pathways of the endocrine organs and therefore impair metabolism, reproduction, and sexual development are known as endocrine disrupting chemicals or EDCs. EDCs cause endocrine disruption by mimicking hormones and can be estrogenic, androgenic, thyroid-disrupting, anti-estrogenic, and/or anti-androgenic. As a result, EDCs can lead to feminization, masculinization, or suppression of both sexes within a species. Although several cases of abnormal biological effects have been reported in Louisiana, and several EDCs have been found in Louisiana water, soil, and air; a bona fide chain of causation documenting actual environmental endocrine disruption has yet to be fulfilled. For example peaks of atrazine as high as 40 mg / liter were reported from the upper Barataria basin, however no detrimental wildlife effects were associated with these reports. On the other hand, documented wildlife effects such as the historical extirpation of brown pelicans from the Louisiana coast in the 1960's were not specifically classified as endocrine disrupting events, but instead thought to be caused by direct bird mortality and disappearance of prey due to endrin contamination. Here, we have compiled a list of preliminary reports and geographic sites that offer an inventory of possible endocrine disrupting events in Louisiana. These cases either warrant more study, or represent sites that should be monitored closely in the future to test whether endocrine disruption is occurring.

Rhea Vidrine

vidrr336@its.nicholls.edu

ph: 985-448-4715

fax: 985-493-2496

29.

Reproductive Disruption of Fishes by Endocrine-Active Wastewater Effluent

Alan Vajda¹, L.B. Barber², A.B. Bolden³, and D.O. Norris³

¹Department of Integrative Biology, University of Colorado, Denver

²U.S. Geological Survey, Boulder, Colorado

³Department of Integrative Physiology, University of Colorado, Boulder

We investigated the impact of wastewater treatment plant (WWTP) effluents on Colorado's Front Range on fish reproduction. WWTP effluents are known to contain endocrine-active compounds including alkylphenols, reproductive steroids, and pharmaceutical contraceptives. Previously, we identified female biased sex ratios, gonadal intersex, asynchronous ovarian development, and other forms of reproductive disruption in feral white suckers (*Catostomus commersoni*) collected downstream of WWTP effluent but not at reference sites.

To investigate the putative link between reproductive disruption observed in feral fish and wastewater effluent, we conducted laboratory experiments with estrogen mixtures and on-site exposure experiments in 2005 and 2006 using a mobile flow-through laboratory. In these experiments, adult male fathead minnows (*Pimephales promelas*) were exposed to either WWTP effluent, reference water from upstream of the wastewater plant, or mixtures of reference water and WWTP effluent. Exposure to diluted wastewater treatment plant effluent significantly elevated vitellogenin and suppressed primary and secondary sex characters.

In 2008, we conducted similar on-site experiments to determine effects of an engineering upgrade (change from trickling filter to activated sludge) on the estrogenicity of the effluent. We report a physiological assessment of changes that have occurred in the endocrine activity (estrogenicity) of the WWTP effluent. We will discuss our integrated hydrological, biological, and chemical approach to assessing anthropogenic impacts on watersheds in North America and Australia.

Alan M Vajda, PhD
Assistant Professor
Dept. of Integrative Biology
University of Colorado
Denver, CO 80217

Alan.Vajda@UCDenver.edu
(720) 939-9871 (mobile)

30.

Mechanisms for Inhibition of Crustacean Molting by Organochlorines: an In vitro Approach

Yanling Meng and Enmin Zou

(Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310, USA)

In order to further understand the mechanism for inhibition of crustacean molting by environmentally persistent organochlorines (OCs), we assayed the effects of five molt-inhibiting OCs, including Aroclor 1242, PCB29, endosulfan, kepone and heptachlor, on NAG mRNA, a biomarker for ecdysteroid signaling, in cultured epidermal tissues from the fiddler crab, *Uca pugilator*. Cultured epidermal tissues were found to be responsive to the molting hormone, with epidermal NAG mRNA being inducible by 20-HE. When molt-inhibiting OCs were administered alone, Aroclor 1242, PCB29, endosulfan and kepone upregulated the expression of NAG gene in cultured epidermal tissues, while heptachlor elicited no effects on the level of epidermal NAG mRNA. Under binary exposure to both 20-HE and an OC, a condition similar to the natural hormonal milieu of epidermal tissues of animals impacted by OCs, both Aroclor 1242 and endosulfan were found to be capable of antagonizing ecdysteroid signaling in cultured epidermal tissues. This antagonizing effect on epidermal ecdysteroid signaling can at least partly explain the inhibitory effects of these two agents on crustacean molting. PCB29, when given together with 20-HE, produced an additive effect on epidermal ecdysteroid signaling but such an additive effect was not observed when kepone was combined with 20-HE, while heptachlor showed no effects on epidermal expression of NAG gene.

Contact Info:

Enmin Zou

em.zou@nicholls.edu

Phone: (985) 448-4711

Fax: (985) 493-2496

31.

Alterations of a Biomarker for Molting in the Brown Shrimp, *Penaeus aztecus*, Subjected to Hypoxia and Sedimentary Naphthalene

Enmin Zou

(Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310, USA)

The brown shrimp, *Penaeus aztecus*, is subject to dual stresses of environmental hypoxia and contamination of polycyclic aromatic hydrocarbons (PAHs) in the northern Gulf of Mexico. The effects of hypoxia and sedimentary naphthalene, administered alone and in combination, on epidermal activity of N-acetyl- β -glucosaminidase (NAG), a biomarker for molt-interfering effects in *Penaeus aztecus*, were investigated. It was found that hypoxia and sedimentary naphthalene, when given simultaneously, significantly inhibited epidermal NAG activity, suggesting that these two environmental stressors together can have adverse effects on molting of the brown shrimp since this enzyme is the end product of ecdysteroid signaling in the epidermis and indispensable for degradation of the exoskeleton. The results of this study also show that sedimentary naphthalene potentiates hypoxia effects on epidermal NAG activity.

Contact Info:

Enmin Zou

em.zou@nicholls.edu

Phone: (985) 448-4711

Fax: (985) 493-2496

32.

Investigation of the Causes of Gonadal Abnormalities in the *Chalcalburnus tarichi*

Guler Unal¹, Emily Marquez¹, Mara Feld¹, Ahmet R. Oguz², Burak Kaptaner² and Ian P. Callard¹

¹Boston University, Department of Biology, 5 Cummington St., Boston, MA 02215

²Yuzuncu Yil University, Department of Biology, Van 65080-Turkey

Abstract: Van City Treatment Plant (CTP) discharges sewage effluent into Van Lake, Turkey. Gonadal abnormalities have been observed in *Chalcalburnus tarichi*, an Cyprinid species endemic to Lake Van Basin, collected from the Van Edremite Gulf of the lake. The rate of occurrence of gonadal abnormalities has been 43.3% and 13.6% for females and males, respectively. Gonadosomatic index is lower and plasma estradiol levels are lower in these fish, compared to fish with developed ovaries. In fish with undeveloped ovaries, the oocyte development was arrested at the beginning of the vitellogenic phase and an increase in apoptotic cells was seen. In fish with undeveloped testes, the testis follicles did not have germ cells and vacuoles were observed in Sertoli cells. Also, vitellogenin was present in the plasma of some male fish collected from some regions of the lake. Currently there are no studies examining the presence of endocrine disrupting chemicals in the effluent of CTP, or in the water and sediment from Lake Van. To determine if there is a relationship between effluent and gonadal abnormalities found in fish, we are cloning aromatase (*cyp19*) and estrogen receptor (ER) genes using *C. tarichi* tissue samples from Turkey. We have a partial sequence of ER β (639 nucleotides) and *cyp19* β (243 nucleotides) cloned from ovary and brain, respectively. In the future, we will measure the level of aromatase and ER expression in fish with normal and abnormal ovaries using quantitative PCR.

Corresponding author: E-mail address: gunal09@bu.edu ; Phone: 1-857-540-2545; Fax: 617-353-6340

33.

A Preliminary Study on Immunohistochemical Detection of Estrogen Receptor in American Alligator Oviducts – Specificity of Antibodies

Ohta, Y.¹, Urushidani, H.², Takeuchi, T.¹, Iguchi, T.², Katsu, Y.³, Kohno, S.⁴, Brandon, M.⁴ and Guillette, L.J.⁴

¹ Department of Veterinary Science, Faculty of Agriculture, Tottori, Japan, ²Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, Okazaki, Japan, ³Laboratory of Reproductive and Developmental Biology, Graduate School of Life Science, Hokkaido University, ⁴Department of Zoology, Florida University, Gainesville, FL, USA

Estrogen is principally important for reproductive function in female vertebrates including reptiles. The majority of actions of estrogen are mediated by specific receptors in the target cells. In American alligators, cDNAs encoding estrogen receptors (ERs) have been cloned, and messenger RNA of ERalpha has been demonstrated in the liver, gonads and other organs. We have recently made an antibody against American alligator ERalpha (Transgenic Inc., Japan). The alligator specific antibody, together with 2 other commercial antibodies against mouse and human ERalpha (MC-20, Sant Cruz Biotechnology, Inc., U.S.A.; Ab-10, Thermo Scientific Anatomical Pathology, U.S.A), was applied to immunohistochemistry for demonstration of ERalpha in the oviduct of juvenile American alligators (2- and 5-year-old). Positive staining of ERalpha was evident in nuclei of epithelial, stromal and muscular cells in all parts of the oviduct, regardless of the type of antibodies. Intensity of staining was stronger in the sections stained with Ab-10 than in those with the alligator specific and MC-10 antibodies. The specificity of ERalpha antibody was verified by immunoblotting using a mammalian cell line (COS-1) expressing alligator and human ERalpha. Alligator ERalpha was recognized by alligator and Ab-10 antibodies, but not by MC-20. Anti-ERalpha antibodies, except MC-20, are useful for immunohistochemical demonstration of ERalpha in the alligator tissues. This work is supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (#21510068).

Contact information:

c/o Yasuhiko Ohta

e-mail: ohta@muses.tottori-u.ac.jp

phone/fax: +81-857-31-5637

34.

Morphological and Molecular Responses in Oviducts of 4 Month-old, FSH-Stimulated Alligators

Brandon C Moore¹, Sara Forouhar², Nicole L. Botteri², Heather J. Hamlin² and Louis. J. Guillette, Jr.²

¹Center for Bioenvironmental Research, Tulane University, New Orleans, LA

²Department of Biology, University of Florida, Gainesville, FL

Here we present data of organism-level responsiveness of immature American alligator oviducts to *in vivo* exogenous gonadotropin stimulation. Four-day, follicle stimulating hormone (FSH) treatments resulted in elevated ovarian aromatase (*CYP19A1*) mRNA levels and circulating estradiol (E2) concentrations. Concomitant with these changes, we observed changes in oviducal epithelial morphology and lumen area, along with alteration of mRNA expression levels of steroid receptors and inhibin/activin signaling subunits. We hypothesize that these alterations were not a direct result of the FSH treatment, but a secondary response caused by the resulting elevation of circulating E2 concentrations. Specifically, we observed increases in oviducal androgen receptor (*AR*) and progesterone receptor (*PR*) mRNA expression levels. These responses mirror those previously reported in magnum of both E2 and FSH-treated, hatchling chickens. Activin β A (*INHBA*) and β B (*INHBB*) subunit mRNA were detected in alligator oviduct, while α subunit (*INHA*) levels were detected at much lower levels. This implies that oviducal activin signaling is present and at much greater levels than inhibin signaling. Lastly, treatment elevated mRNA expression levels of the activin inhibitory protein, follistatin (*FST*). This indicates an oviducal responsiveness of this TGF β signaling system even in immature oviducts. Taken together, these results indicate that immature alligator oviduct is an endocrine responsive tissue and androgens, progestins, and activin signaling are putatively involved in regulation of oviducal function.

Brandon Moore

bmoore2@tulane.edu

O: 504-862-8454

Fax: 504-988-6428

35.

Effects of Embryonic Exposure to Dieldrin and *p,p'*-DDE on Endocrine Mediated Signaling in the *Danio rerio* and *Trachemys scripta* Models

Mara H. Feld¹, Giuseppe Cullaro¹, Emily Marquez¹, Guler Unal³, Pericles Stavropoulos², Ian P. Callard¹

¹Department of Biology, Boston University, Boston MA 02215

²Department of Chemistry, Missouri University of Science and Technology, Rolla MO 65409

³Department of Biology, Yuzunca Yil University, Van Turkey

Early developmental exposure to environmental endocrine disrupting chemicals (EDCs) is likely to be related to reproductive system disorders and hormone sensitive cancers in humans. Previous studies have shown reproductive abnormalities in turtles (*Chrysemys picta*) inhabiting a pond (Moody Pond) contaminated with groundwater plumes originating from the Massachusetts Military Reservation (MMR) Superfund site, relative to turtles from a reference site (Washburn Pond). Analysis using Gas Chromatography/Mass Spectrometry (GC/MS) showed that while dieldrin was detected only in *C. picta* egg yolk from Moody Pond animals (59 ug/kg), *p,p'*-DDE (dichlorodiphenyldichloroethylene) was detected from both Moody Pond (51 ug/kg) and the reference site (177 ug/kg). Before examining the relationship between pesticide exposure and endocrine disruption in the turtle model, we first screened these compounds in a rapid *Danio rerio* embryo test system. We have utilized quantitative reverse-transcription PCR (QPCR) to characterize pesticide induced changes in estrogen-, androgen-, and aryl hydrocarbon receptor-regulated signaling. Neither *p,p'*-DDE or dieldrin significantly altered Androgen Receptor (AR), vitellogenin, Estrogen Receptor (ER), or aromatase-A and -B gene expression, although aromatase-A did appear to be slightly reduced by dieldrin in a dose-dependent manner. Exposure to dieldrin or *p,p'*-DDE significantly induced cytochrome P450 1a1 gene expression (2- to 4-fold) in the *D. rerio* embryo model. We are currently examining the effects of dieldrin and *p,p'*-DDE in a *Trachemys scripta* egg painting model. Data collection, including gene expression changes in the gonad, kidney, adrenal gland, and liver of neonatal *T. scripta*, are currently ongoing and will be reported in the future.

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mhfeld@bu.edu

(608) 957-2763 Cell

(617) 353-6340 Fax

36.

Epithelial Morphology of the American Alligator Phallus

Ketan K. Mathavan, Brandon C. Moore, and Louis J. Guillette, Jr.

Department of Biology, University of Florida, Gainesville, FL

Male American alligators living in certain polluted, Florida lakes have been reported to have a smaller phallus than those living in uncontaminated lakes. As a sentinel species, it is important to understand the origin and extent of these genital abnormalities. Thus far, research has established EDCs as a potential cause of an array of abnormalities seen in these animals, including phallus abnormalities. Our research examines and provides baseline standards for normal morphology of the alligator phallus. Using differential histological staining, various morphologies were identified. We observed two different epithelia bounding the phallus. A stratified, squamous epithelium composed the majority of the surface, whereas the remaining epithelium was ciliated and stratified, columnar. Both epithelia were histochemically reactive. The former showed positive periodic acid-Schiff (PAS) and Alcian blue (AB) staining in the form of a gradient, increasing from basal to superficial cells. The latter showed positive PAS, AB, and periodic acid-methenamine silver (PAMS) staining in columnar cells. Based on the histochemical specificities and morphology of the epithelium, the squamous epithelium is likely used for protection from pathogens and mechanical damage. Likewise, the ciliated epithelium may facilitate the translocation of sperm along the medial sulcus during intromission. Further research will determine the phallus morphology of affected males, providing a greater understanding of the effects of EDCs, as well as, suggest developmental differences between affected and unaffected male alligators.

Ketan K. Mathavan
2025 NE 15th St.
Gainesville, FL 32609
Cell: (772) 284-5423
Email: kkmathavan@gmail.com

37.

Environmental Influence on Yolk Steroids in American Alligators (*Alligator mississippiensis*)

Hamlin, H.J., Albergotti, L.A., Mutz, J, Guillette, L.J.Jr.

Department of Biology, 223 Bartram Hall, PO Box 118525, University of Florida,
Gainesville, Florida, 32611-8525 USA

Maternal transmission of developmental resources is a critical factor in offspring survival and fitness. Hormones, in particular, are important regulators of embryonic development and prenatal hormone exposure can have considerable impacts on nearly every aspect of early life. The inability of the embryo to produce and regulate its own hormone milieu early in development necessitates reliance on maternal contributions. Concentrations of steroid hormones were measured in the yolk of alligator eggs collected from an agriculturally contaminated location (Lake Apopka), an area of industrial contamination (e.g. heavy metals) (Kennedy Space Center, KSC) and a reference locale (Lake Woodruff) in Florida. Yolk was sampled at both pre (stage 12) and post (stage 24) sex determination time points. Progesterone concentrations were significantly higher from yolk collected at Lake Woodruff at stage 12 versus the other two locations. Estradiol was significantly lower from yolk collected from KSC. There were no differences in yolk testosterone at stage 12 for any location. All hormones were significantly lower at stage 24 as compared to stage 12, but none of the stage 24 samples from any location differed significantly from each other. Environmental conditions leading to altered concentrations of yolk steroids could cause a host of developmental and reproductive dysfunctions, many of which have already been associated with alligators living in contaminated lakes in Florida.

H.J. Hamlin
University of Florida
Telephone: 352-392-1098
Fax: 352-392-3704
Email: hjhamlin@ufl.edu

38.

**A Novel Approach to the Investigation of Environmental Endocrine Disruptors:
Ecotoxicogenomics in the American Alligator (*Alligator mississippiensis*)**

Scott M. Clayman¹, Satomi Kohno¹, Ashley S. P. Boggs¹, Russell H. Lowers² and Louis J. Guillette Jr.¹

University of Florida, Department of Biology, Gainesville, FL¹, Dynamac Corporation,
Kennedy Space Center, FL²

The issue of endocrine disruptors has been a global concern. Lake Apopka, located in central Florida, is heavily contaminated with the pesticides dicofol and DDT, which can alter the endocrine system. Previous studies reported alligators from Lake Apopka displaying reproductive abnormalities, small phalli in males, and ovarian multiocytic follicles in females. We have developed a novel approach to survey the wildlife with a molecular marker (mRNA expression) using the blood cells in American alligator. This would allow us to run ecotoxicogenomics analyses without sacrificing the animal even if the animal is endangered and to further understand how the environment impacts these wildlife. The alligators in our study were caught at a reference site (Lake Woodruff National Wildlife Refuge, DeLeon, FL), a site contaminated with agricultural chemicals (e.g., DDE and other OCs) (Lake Apopka, Apopka, FL) and a site contaminated with industrial chemicals (e.g., heavy metals, PCBs) (Merrit Island National Wildlife Refuge at NASA, Kennedy Space Center, FL). Total RNA was isolated from whole blood cells and mRNA expressions were analyzed by quantitative real-time PCR. Estrogen receptor beta (ESR2) mRNA expression at Lake Apopka was significantly different with two orders of magnitude greater expression compared to Lake Woodruff and NASA. Interestingly, a previous study showed ovarian ESR2 mRNA expression of juvenile alligators in Lake Woodruff was greater than in Apopka. Although the functions of ESR2 in the blood cells are unknown, this study suggests that ESR2 mRNA expression in blood cells may be a sensitive marker of environmental contaminants exposure.

Scott.Clayman@gmail.com

Phone (904) 868-0290

Fax (352)392.3704

39.

**The Medullary Rest:
A Naturally Occurring Intersex Region of American Alligator Ovary**

Nicole L. Botteri, Brandon C. Moore, Heather J. Hamlin, Ashley N. Lawler, Ketan Mathavan, and Louis J. Guillette, Jr.

Department of Biology, University of Florida, Gainesville, FL

In the American alligator, temperature dependent sex determination (TSD) directs gonadal differentiation during embryonic incubation and is characterized as being an absolute process, producing either an ovary or a testis. However, in depth histological investigation of alligator ovary reveals a naturally occurring intersex region. The “medullary rest”, first described by T.R. Forbes in the 1930s, is an atypical ovarian region lacking cortical and medullary morphology characteristic of an alligator ovary. In contrast, the medullary rest is comprised of disorganized tubule-like structures containing presumptive germ cells, reminiscent of testicular tissue. Here, we further the investigation of this region started by Forbes by using differential histological stains to show that the medullary rest is a *dynamic region*, maturing throughout ontogeny, demonstrating *both ovarian and testicular* characteristics. Future endeavors will characterize tissues of the medullary rest using sexually dimorphic immunohistochemical markers and quantify potential hormonal responsiveness. Characterization of the medullary rest is essential in order to ascertain the effects of EDCs on this region, and subsequently, to predict potential ramifications for reproduction.

Nicole L. Botteri
PO Box 118525
Gainesville FL, 32611-8525
Phone: (352) 392-1098
Fax: (352) 392-3704
Cell: (352) 359-2940
Email: nbotteri@ufl.edu

40.

Diagnostic Thyroid Histology of Neonatal American Alligators

Ashley S. P. Boggs¹, Eugenia Timofeev¹, Ketan Mathavan¹, Louis J. Guillette Jr.¹

1. University of Florida, Department of Biology

Thyroid histology can be used as a diagnostic tool for detection of thyroid disruption. Recent papers describing protocols for quantifying thyroid disruption in mice and frogs exposed to thyroid disrupting chemicals serve as excellent templates for detection. However, species and age can alter criteria. Thyroids from neonatal American alligators from three sites: 1) a highly contaminated site, Lake Apopka, 2) a reference site, Lake Woodruff National Wildlife Refuge, and 3) a site high in PCBs but unstudied for thyroid morphology, Merritt Island National Wildlife Refuge, were examined by combining the quantification techniques of Grim et al (2009) and Hooth et al (2001). This approach provided markers of thyroid disruption in a new species, the American alligator, while maintaining the ability for cross species comparisons. Area of the thyroid gland, follicular cell (FC) hypertrophy and FC hyperplasia, and colloid depletion were quantified using the criteria of Grim et al. Severity of hyperplasia was quantified by the criteria of Hooth et al. Initial results indicate that FC hypertrophy is not a marker of disruption in neonatal alligators, while the area and severity of FC hyperplasia better indicate disruption. Novel techniques for detecting disruption include determination of follicular lumen shape by three-dimensional imaging and labeling of colloid condition by Masson's modified trichrome and periodic acid-Schiff. Alcian blue was used to identify C cells previously undocumented in alligators. This study describes the morphology of neonatal American alligator thyroid glands and provides evidence of thyroid disruption by combining established and novel techniques.

Ashley Boggs
boogsta@ufl.edu
University of Florida
Department of Biology
PO Box 118525
Gainesville, FL 32611

41.

Exploring Steroidogenesis in the Chorioallantoic Membrane of the Domestic Chicken (*Gallus gallus*) and the American Alligator (*Alligator mississippiensis*)

Lori C. Albergotti¹, Heather J. Hamlin¹, Michael W. McCoy^{1,2}, and Louis J. Guillette, Jr.¹

¹Department of Biology, University of Florida, Gainesville, FL,

²Department of Integrative Biology, University of South Florida, Tampa, FL

During development, amniotes (mammals, reptiles, and birds) form extraembryonic membranes, which function in nutrient and gas exchange, waste removal and shock absorption. In live-bearing amniotes, extraembryonic membranes and maternal uterine tissues contribute to the placenta, an endocrine organ that synthesizes, transports, and metabolizes hormones. Surprisingly, the endocrine role of extraembryonic membranes has not been investigated in oviparous (egg-laying) amniotes despite similarities in their basic structure and function. To begin addressing this question we are exploring steroidogenesis in the chorioallantoic membrane (CAM) of the domestic chicken (*Gallus gallus*) and the American alligator (*Alligator mississippiensis*). In the chicken and alligator CAM, we quantified mRNA expression of steroidogenic enzymes and steroid receptors and performed protein immunolocalization of the progesterone receptor (PR) and estrogen receptor α (ER α). We found that the CAM expresses mRNA for all of the steroidogenic enzymes and steroid receptors examined to date, and some of these display altered expression with developmental stage. Further, we show that the chicken CAM synthesizes progesterone *in vitro* in the presence of cholesterol. Our data indicate that the CAM is steroidogenic and suggests that endocrine activity of extraembryonic membranes is not a novel characteristic of placental amniotes. Despite the localization of endocrine disrupting contaminants (EDCs) in the CAM, extraembryonic membranes are not established as targets of endocrine disruption. Our data show that the CAM expresses PR and ER and thus is a potential target of EDCs. We are investigating the endocrine role of extraembryonic membranes to better understand the impact of EDCs on these tissues.

Lori C. Albergotti

Email: lalberg@ufl.edu

Phone: 352-281-2786

Fax: 352-392-3704