RELATIVE SENSITIVITY TO DIOXIN AND/OR CO-PLANAR PCBs

Chicken > Pheasant > Gull > Bald Eagle

Jungle Crow?

Common Cormorant?

Wood Duck?

- Can be predicted with hepatocyte cultures
- Might be very easy to predict with other *in vitro* approaches that we are thinking about

Toxicogenomics

- The study of relationships between the genome (the cellular complement of genes) and the adverse effects of toxicants
- Includes studies on the effects on the genes (mutations) and the effects on gene expression (mRNA *and* proteins)
- Most, if not all, toxic effects of chemicals involve changes in gene expression
- Can be used to discover novel effects of endocrine disrupting chemical (EDCs)

The Promise of (Eco)Toxicogenomics

- Beginning to be seen for basic research (e.g. mechanisms of action of toxicants) and for questions involving human health
- A large and growing interest among ecotoxicologists
- Promise for ecotoxicology also becoming realized

"Closed" and 'Open" Methods for mRNA Expression Analysis

Closed (e.g., DNA Microarrays):

- Require DNA sequence information for the species of interest (with some important exceptions)

Open:

- No DNA sequence information from a species is needed to identify potential "hits" (candidate genes)

Eco-toxicogenomics at the National Wildlife Research Centre (NWRC)

- For now, we are concentrating on ‘Open’ rather than ‘Closed’ methods for measuring mRNA expression

"Open" mRNA Expression Methods Currently Used at NWRC

- **Fluorescent RNA Arbitrarily Primed PCR (FRAP-PCR)**
  - **Crump,Chiu,Trudeau & Kennedy -- Submitted for Publication**
- **Serial Analysis of Gene Expression (SAGE)**
  - **"Long-SAGE" (21 base-pair tags)**
  - **Jones, McArthur, Kennedy *et al.* -- Manuscripts in Preparation**
  - **Quantitative PCR (Q-PCR) is used to confirm findings that are obtained using FRAP-PCR and SAGE**

Current Studies

1. Effects of PBDEs on mRNA expression in primary cultures of herring gull neuronal cells (FRAP-PCR and Q-PCR)
2. Effects of TCDD on mRNA expression in primary cultures of chicken hepatocytes (SAGE and Q-PCR)

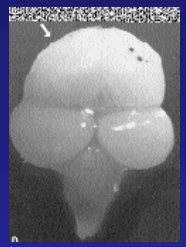
Polybrominated Diphenyl Ethers (PBDEs)

- Additive flame retardants
- Persistent and bioaccumulative
- Toxicological impacts of exposure relatively uncharacterized
- ↓ levels of nicotinic receptors and alters neurobehavioural endpoints
- ↓ circulating T4 levels

Monitoring program has provided valuable data on contaminant trends in Great Lakes herring gulls

PBDEs in egg homogenates

Norstrom *et al.* 2002



PBDEs in herring gull brains

- ΣBDE 47, 99, 100 detected at levels ranging from 10 - 96 µg/kg wet weight

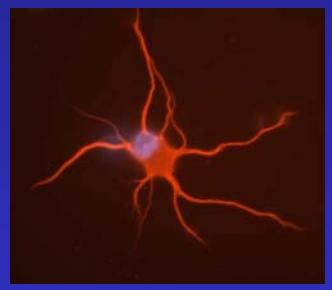
- What type of research should be conducted?
- Which endpoints?
- Target tissues?

Avian embryonic neuronal cell culture

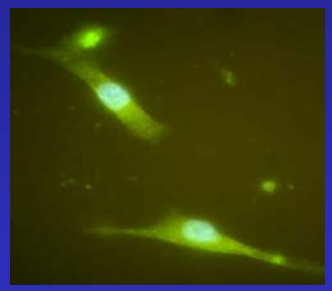
(Crump, Jagla, Kennedy (2006) submitted)

- Herring gull and chicken have been tested to date
- Cell culture characterization

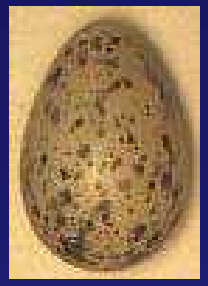
Glial cell

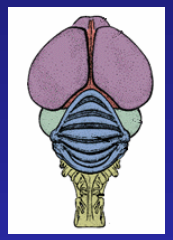


Neuronal cells



- Controlled dosing experiments with Bromkal 70 to assess changes in gene expression and cell viability



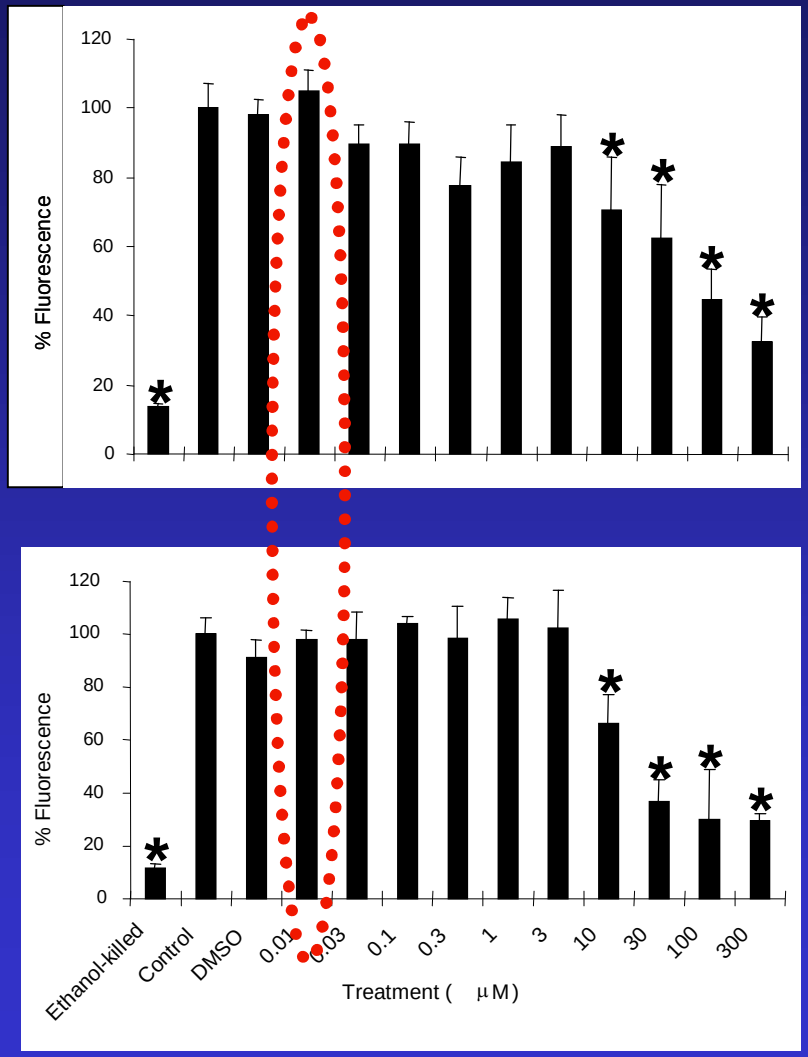


1. Chicken or herring gull embryos are sacrificed at mid-incubation (Day 11 or 14, respectively)
2. Harvest the cerebral hemispheres and prepare primary neuronal cells
3. Plate cells; incubate at 37 ° C for 24 h
4. Dose cells with Bromkal 70 in DMSO
5. Incubate for 24 h

Cell viability
(Calcein-Am)

RNA Isolation
(gene expression analysis)

Cell viability



Chicken neuronal cell viability

- no effect of DMSO vehicle control
- [Bromkal 70] >10 µM ↓ viability
- * – $p<0.05$

Herring gull neuronal cell viability

- similar results as chicken

Fluorescent RNA Arbitrarily Primed-PCR (FRAP-PCR)

- “Open” mRNA expression method
- No DNA sequence information from a species is needed to identify potential “fingerprints”

Identification of novel gene targets responsive to Bromkal 70 exposure in herring gull neuronal cells

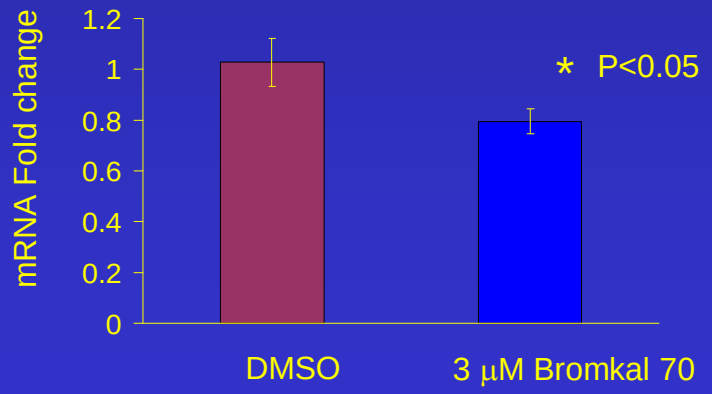
Increased expression

- **Transcription factor 4**
- **Mitochondrial elongation factor**

Decreased expression

- **SET-binding protein**
- **Ten-M3**
- **Polyubiquitin**

Validation of SET-binding protein expression by Q-PCR



FRAP-PCR Highlights

GenHunter Method

- Many gene target hits were anchored to the 3'-untranslated region (UTR) and were unidentifiable
- 10-20% success rate for identification of functional mRNA sequences

FRAP-PCR Method

- 80-90% of gene targets are within the coding region
- Identification of gene function and mechanism enhanced
- Method development is completed – Crump *et* al. manuscript submitted
- Non-radioactive, efficient, straightforward, amenable to use with various species

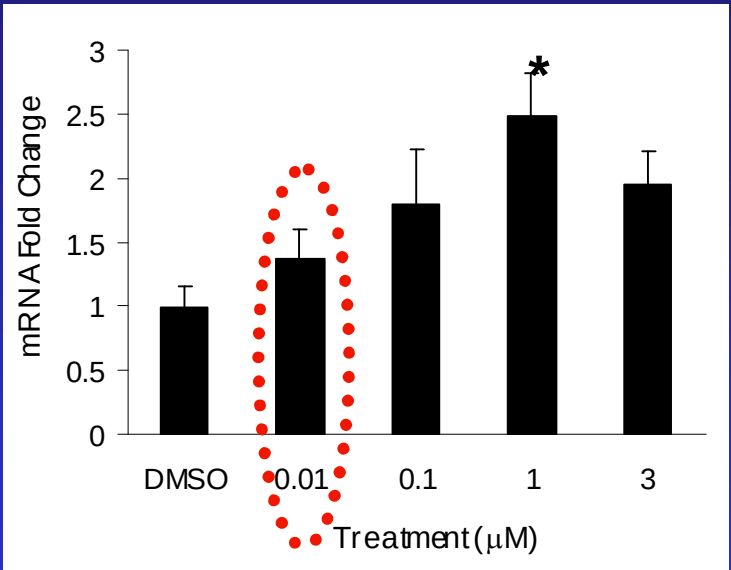
Everyone likes a hypothesis!

Development of Q-PCR assays

1. Nicotinic acetylcholine receptor (nAChR) α-7

2. TR α and β

Results:



- Herring gull nAChR α-7 mRNA increased in a dose-dependent manner to a peak of 2.5-fold induction at 1 μM Bromkal 70 (* - $p<0.01$)

Conclusions

1. First study to assess the effects of PBDEs on gene expression in herring gulls
2. FRAP-PCR identified novel endpoints in an avian species with limited genetic information
3. Bromkal 70 increased expression of nAChR α-7 and TR α mRNA
4. Neuronal cell culture enhances our ability to identify potential effects of contaminant exposure

Future goals

- Use neuronal cell culture to assess effects of other suspected neurotoxins
- Compare *in vitro* and *in vivo* effects
- Compare RNA fingerprints of gulls from various colonies – contaminant/geographic signatures?

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The Principles of Serial Analysis of Gene Expession (SAGE)

1. A short tag of mRNA (21 bp) contains sufficient information to uniquely identify a transcript ***provided that the tag is obtained from a unique position of the transcript*.**
2. The tags (after conversion to cDNA) are linked together to form series of tags that are cloned and sequenced.
3. Quantification of the number of times that a particular tag is observed provides an expression level of the corresponding mRNA transcript.
4. An 'Open' method – no need for DNA sequence information of a species to identify differences in tag numbers in treated vs. control cells

Gene Expression Using SAGE

5’

GCGTTTTCCAGGGCTTCCCAGAGGTCTGTGCGACTAGCCCCTGTCTATCAAAAG
TTATTAGAGAGGATGAAGCATTAGCTTGAAGCACTACAGGAGGAATGCACCAC
GGCAGCTCTCCGCCAATTTCTCTCAGATTTCCACAGAGACTGTTTGAATGTTTTC
AAAACCAAGTATCACACTTTAATGTACATGGGCCGCACCATAATGAGATGTGAG
CCTTGTGCATGTGGGGGAGGAGGGAGAGAGATGTACTTTTTAAATCATGTTCCC
CCTAAACATGGCTGTTAACCCACTGCATGCAGAAACTTGGATGTCACTGCCTGA
CATTCACTTCCAGAGAGGACCTATCCCAAATGTGGAATTGACTGCCTATGCCAA
GTCCCTGGAAAAGGAGCTTCAGTATTGTGGGGCTCATAAAACATGAATCAAGCA
ATCCAGCCTCATGGGAAGTCCTGGCACAGTTTTTGTAAAGCCCTTGCACAGCTG
GAGAAATGGCATCATTATAAGCTATGAGTTGAAATGTTCTGTCAAATGTGTCTCA
CATCTACACGTGGCTTGGAGGCTTTTATGGGGCCCTGTCCAGGTAGAAAAGAAA
TGGTATGTAGAGCTTAGATTTCCCTATTGTGACAGAGCCATGGTGTGTTTG
TAATAATAAGGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

3’

A Genetic Barcode

SAGE Logic

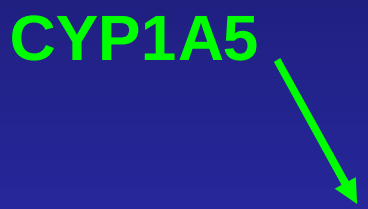
Number of Tags (each tag is CATG + 17 bp)

	Un-Treated	Treated
Tag A	10	14
Tag B	8	11
Tag C	21	16
Tag D	**5**	**56**
Tag E	**21**	**2**

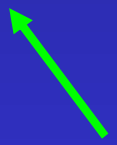
Pilot SAGE Study

- Cultured chicken embryo hepatocyte (CEH) cells (24-hour exposure to TCDD (multiple doses)
- Conducted SAGE analysis of 1nM dose

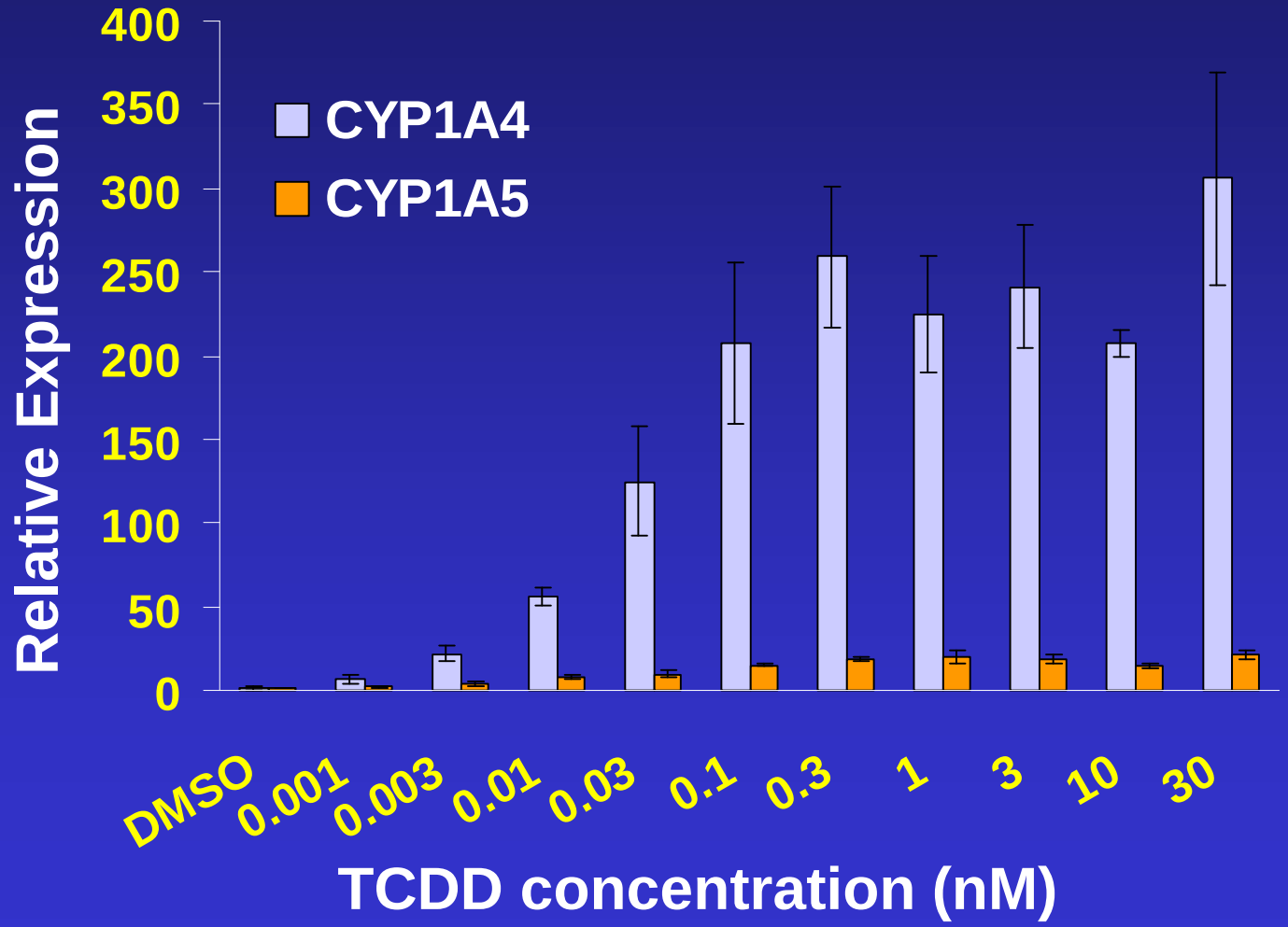
Number of SAGE-tags



	Control	TCDD-treated
CATGCAATAAACAAAAGCCAT	4	7
CATGCAGGGAATCCCTCAGCG	0	22



CYP1A mRNA Induction in CEH Cultures
(Q-PCR Analysis)



Several Other Interesting “Hits”

For example:

- *Fatty Acid Biosynthesis downregulation*
- *Uroporphyrinogen decarboxylase downregulation*
- *Type 2 Diabetes Associated Genes*
- *Genes associated with embryonic deformities (eye and beak)*
- *Apolipoprotein A1 downregulation*

SAGE Data Analysis

- We are using the Genomic Model Organism Database (GMOD) servers of the Marine Biological Laboratory (MBL), Woods Hole, MA
- The GMOD Project:
  - largely open source
  - developing a complete set of software for creating and administering model organism databases
  - 23 projects, 11 of which are SAGE projects
- Dr. Andrew McArthur, Marine Biological Laboratory, Woods Hole, MA

http://www.gmod.org/

General Comments on SAGE

- *Many novel transcipts are being identified in mouse and human*
- *SAGE data available on the Web is increasing being 'mined' to discover disease-associated genes*
- *Enormous potential for the discovery of novel effects of toxic chemicals*
- *Technically challenging – need good skills 'at the bench'*
- *Powerful bioinformatics tools are now available, and improvements are being developed*
- *A challenging area to work in – most people use microarrays*
- *Utility for wild species – to be determined*

The Challenges for Applying New 'Omics' Technologies to Wild Species

- **Scientific – some similarities, but important differences**
  - **Many species**
  - **Concern often for populations, not individuals**
- **Technical – Very challenging problems**
  - **Microarrays of use, but is it really efficient for each laboratory to develop a microarray for its favorite species??**
  - **Can other approaches be exploited and developed?**
  - **Will new "Open" methods for studying mRNA expression be of general use?**
- **Financial – Limited resources**

COORDINATED INTERNATIONAL RESEARCH PROGRAM on TOXICOGENOMICS