Biomarkers for Endocrine Disruption in Feral Fish from the U.S.

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National Wetlands Research Center
Lafayette, Louisiana

U.S. Department of the Interior
U.S. Geological Survey

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USGS. A multi-disciplinary science organization that focuses on biology, geography, geology, geospatial information, and water. Dedicated to the timely, relevant, and impartial study of the landscape, our natural resources, and the natural hazards that threaten us.

BRD Mission. Provide the scientific understanding & technologies needed to support the sound management & conservation of our Nation's biological resources.

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Lafayette, LA
The long-term goals:
- to describe the status and trends in the quality of a large, representative part of the Nation's surface- and ground-water resources
- to provide a sound, scientific understanding of the primary factors affecting the quality of these resources.
BEST is the only long-term monitoring program documenting biological effects resulting from contaminants at broad temporal and geographical scales.
Methods Used in BEST

- Cytochrome P450-1A induction
- Necropsy-based fish health assessments
- Histopathological assessments
- Immune system indicators
- Condition factor and GSI
- OC and elemental analysis of tissue
- Reproductive and endocrine bioindicators
Type Chemical Examples

1. anti-estrogenic: PCBs
2. estrogenic: BDEs, artificial estrogen
3. anti-androgenic: pp-DDE
4. androgenic: some pharmaceuticals and personal care products
Halogenated Compound Classes
PBDEs DDTs PCBs
BDE71 Trifluralin Oxyfluorfen p,p-DDE PCB101
BDE47 Benfluralin trans-Nonachlor p,p-DDD
PCB110
BDE66 Tefluthrin Tetradifon p,p-DDT PCB146
BDE100 Desulfurylfipronil Cyhalothrin PCB118
BDE99 Chlorpyrifos Cyfluthrin PCB138
BDE85 DCPA Pentachloronitrobenzene PCB149
BDE154 Oxychlorodane Hexachlorobenzene (HCB)
PCB187
BDE153 Fipronil_Sulfide Pentachloroaniline (PCA)
PCB183
BDE138 Fipronil Octachlorostyrene PCB174
BDE183 trans-Chlordane Triclosan PCB180
Endosulfan_I Pentabromotoluene PCB194
cis-Chlordane Triclosan_methoxy PCB206
cis-Nonachlor Fire_master PCB177
Dieldrin Dechlorane_plus PCB170
PCB151
Organochlorine Pesticides
Anthropogenic Waste Indicators (AWIs)
cumene atrazine
bromoform pentachlorophenol
phenol 4-octylphenol
1,4-dichlorobenzene tri(2-chloroethyl phosphate)
d-limonene anthracene
acetophenone diazinon
para-cresol carbazole
isophorone caffiene
camphor tonalide (AHTN)
isoborneol OPEO-1
menthol 4-cumylphenol
methyl salicylate bromacil
isoquinoline galloxide (HHCB)
dichlorovos metalaxyl
2-methyl-naphthalene anthraquinone
indole metolachlor
3,4-dichlorophenyl NPEO1-total
1-methyl-naphthalene chlorpyrifos
skatol triclosan
2,6-dimethyl-naphthalene bisphenol A
butylated hydroxyanisole (BHA) OPEO-2
5-methyl-1H-benzotriazole NPEO2-total
N,N-diethyl-meta-toluamide (DEET)
tri(dichloroisopropyl) phosphate
4-tert-octylphenol triphenyl phosphate
benzophenone tris (2-butoxyethyl phosphate)
tributylphosphate benzo(a)pyrene
ethyl citrate 3-beta-coprostanol
cotinine cholesterol
para-nonylphenol beta-sitosterol
Pharmaceuticals
Cotinine Diphenhydramine
Albuterol Diltiazem
Acetaminophen Carbamazapine
Ranitidine Fluoxetine
1,7-dimethylxanthine Dehydronifedipine
Codeine Warfarin
Caffeine Miconazole
Trimethoprim Erythromycin
Thiabendazole Azithromycin
Sulfamethoxazole Cimetidine
Results

Halogenated Compounds in Fish Tissues

Concentration (ng g⁻¹)

brain, fillet, liver, stomach, gonad

PCBs, DDTs/10, OC Pest, PBDEs
Biomarkers at Varying Levels of Biological Organization

Endocrine function: liver, hypoth., gonad

Genetics: diversity, development

Reproductive condition: sex steroids, GSI, Vtg

Together, provide more power, confidence that biologically relevant assessments are made.
Biomarkers at Varying Levels of Biological Organization

- Genetic
- Biochemical
- Physiological
- Behavioral
- Immunological
- Bioenergetic
- Populations

Increasing Integration and Ecological Relevance
Reproductive Biomarkers

1. GSI
2. VTG
3. Gametes (egg)
4. Sex Steroids
   - Estradiol: egg growth & development
   - 11-KT: spermatogenesis
   - Testosterone: precursor for E & KT
   - Progesterone: egg maturation
Biological responses to environmental stressors (biomarkers) are quantifiable and predictive of ecosystem health.

In complex mixtures, with nutrient and temperature variabilities, can biomarkers yield useful information?

Biomarker: a xenobiotically induced alteration in cellular or biochemical components or processes, structures or functions that is measurable in a biological system or sample

Hypotheses: Are the populations of animals showing differences among sites?
Razorback Suckers (*Xyrauchen texanus*)
Common Carp
(Cyprinus carpio)
Common Carp (Cyprinus carpio)
Extracting Milt from Carp Testis
Population Growth for Las Vegas Valley

1950
Pop 47,000

1960
Pop 116,000

1970
Pop 262,000

1980
Pop 444,000

1990
Pop 708,000

2000
Pop 1,300,000
Lower Colorado R. Study Sites

Largescle Suckers (Catostomus macrochelius)
Cellular Structures

Quality Assessment Assays

**Acrosome** (sturgeon, oysters, mammals)

**Lectin**

**Nucleus**

**Plasma membrane**

**Viability staining**

**DNA staining**

e.g., Comet assay, SCSA, necrosis

**Genetic integrity**

**Percent:** Fertilization

**Hatching**

**Development**

**Mitochondria**

**Axoneme**

**Cytoplasm**

**Rhodamine 123**

**Flagella**

**Motility estimates**

**CASA**

**Morphology**

**Percent: Fertilization**

**Hatching**

**Development**

**Cellular Structures**

**Quality Assessment Assays**
Studies on Spermatozoa

- Quantifies male reproductive function
- Genetic resource
- Various cell/molecular parameters reflect reproductive capabilities
Overview Flow Chart: Sperm Cell Quality

Collect Carp (March 06-08, n=13/site)
Collect Razorback suckers (n=11, '08; n=15, '09)

Overnight shipment to lab

Study milt.....
Male Carp GSI: Lake Mead 2006

- Boulder Basin: 5.1
- Gregg Basin: 5.4
- Las Vegas Bay: 5.6
- Overton Arm: 7.1
- Willow Beach: 4.6
Male GSI: Lake Mead 2007

$P < 0.0001$

OA = LVB > LVW = WB

Las Vegas Wash | Las Vegas Bay | Willow Beach | Overton Arm
--- | --- | --- | ---
4.4 | 6.4 | 4.1 | 7.4
Razorback Sucker Condition Factor ’08-09

Razorback Condition factor

<table>
<thead>
<tr>
<th>NS</th>
<th>2008</th>
<th>2009</th>
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<tr>
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<td>1.4</td>
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<tr>
<td></td>
<td>1.6</td>
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</table>

Las Vegas Bay
Echo Bay + Overton Arm

2008
2009

USGS: science for a changing world
con’t. Flow Chart: Sperm Cell Quality

Prepare slide for later morphology assessments by brightfield and image analysis

↓

Perform visual motility assessments by darkfield microscopy; take aliquot for computer assisted sperm analysis (CASA)

↓

Flow cytometry for membrane integrity (viability), mitochondrial function (mito membrane potential), apoptosis (programmed cell death).

↓

Fix cells for later, sperm counts per mL milt, and DNA integrity (fragmentation)
Razorback Sucker: Sperm Morphology

**Cellular Morphology**

<table>
<thead>
<tr>
<th></th>
<th>2008</th>
<th>2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Las Vegas Bay</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Echo Bay + Overton Arm</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

**Abnormal (%)**

**NS**
Assessment of Motility

The most commonly used quality assessment.

Variable correlations with fertility in other species.

Fueled by mitochondria.

WHO: morphology, motility, counts
Motility by Darkfield Microscopy
Motility by CASA: Computer Assisted Sperm Motion Analysis

curvilinear velocity
average path velocity
linear velocity
linearity
beat – cross frequency
amplitude lateral head displacement

total motility
progressive motility
Motility: 2006

\[ P < 0.0001 \text{ Total} \]
\[ P = 0.0080 \text{ Prog.} \]

- Boulder Basin
- Gregg Basin
- Las Vegas Bay
- Willow Beach
- Overton Arm

Total: OA, GB, BB > LVB, WB
Prog.: OA, GB > LVB, WB
Motility: 2007

Total & Prog. LVB=LVW=WB=OA
Motility: 2008

$P < 0.0001$ Total
$P < 0.0001$ Prog.

Motility 2008

Total & Prog. OA > LVB > LVW > WB
Razorback Sucker: “Visual” Motility

Sperm Motility by Microscopy

- Las Vegas Bay
- Echo Bay + Overton Arm

Motility (%)

Duration (s)

- 08 motility
- 09 motility
- 08 duration
- 09 duration
Razorback Sucker: “Visual” Motility

Visual Motility: 2008 + 2009

Motility (%)

- 100
- 90
- 80
- 70
- 60
- 50
- 40
- 30
- 20
- 10
- 0

Duration (s)

- 60
- 50
- 40
- 30
- 20
- 10
0

Las Vegas Bay
Echo Bay + Overton Arm

Motility
Duration

NS
Sperm Counting by Flow Cytometry

Fluorescence

Size

Cells

Beads
Carp Sperm Counts: 2007

\[ P = 0.027 \]

2007

```
OA > WB > LVB > WB
```
Carp Sperm Counts: 2008

NS

1.0E+10 -
9.0E+09 -
8.0E+09 -
7.0E+09 -
6.0E+09 -
5.0E+09 -
4.0E+09 -
3.0E+09 -
2.0E+09 -
1.0E+09 -
0.0E+00 -

Sperm/mL milt

Las Vegas Wash | Las Vegas Bay | Willow Beach | Overton Arm

0.0E+00
Razorback Sucker: Sperm Counts

<table>
<thead>
<tr>
<th>Year</th>
<th>Las Vegas Bay</th>
<th>Echo Bay + Overton Arm</th>
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<tbody>
<tr>
<td>2008</td>
<td>5.00E+09</td>
<td>3.00E+09</td>
</tr>
<tr>
<td>2009</td>
<td>6.00E+09</td>
<td>4.00E+09</td>
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</table>
Membrane Integrity and Mitochondrial Membrane Potential

- green = better
- red = not so good

10K cells, 3x/fish
Membrane Integrity: Viability

Razorback Sucker

Carp
Carp Viability and Mitochondrial Function: 2007

$P = 0.0056$ mito
NS viab

LVW=LVB=OA>WB
Carp Mitochondrial Function: 2008

$P = 0.0017$

OA > LVW > WB
Razorback Sucker: Viability and Mitochondrial Function

Razorback Sucker (2008+2009)
Apoptosis

Occurs prior to mitochondrial dysfunction and cell death

Red

Green
Annexin-V binding
Carp Apoptosis 2007, 2008

2007

WB > OA = LVW = LVB

$P < 0.0001$

2008

WB > LVW > OA

$P < 0.046$
Gating for Nuclear DNA Fragmentation
2006 and 2007: Percent Fragmented DNA

NS, P = 0.0788

2006

P = 0.0009

2007

WB ≥ LVW ≥ LVB = OA
# Site Differences in Carp Sperm Cell Quality

<table>
<thead>
<tr>
<th></th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
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<tr>
<td>Morphology</td>
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<tr>
<td>Viability</td>
<td></td>
<td></td>
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<tr>
<td>Mito function</td>
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<td>⭐</td>
<td>⭐</td>
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<tr>
<td>Apoptosis</td>
<td>⭐</td>
<td>⭐</td>
<td>⭐</td>
</tr>
<tr>
<td>Sperm count</td>
<td>⭐</td>
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<td></td>
</tr>
<tr>
<td>DNA fragmentation</td>
<td>⭐</td>
<td></td>
<td>in analysis</td>
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</table>

*USGS*
## Carp Tissue 2006

### Carp male composites contaminants (ng/g ww)

<table>
<thead>
<tr>
<th>Site</th>
<th>Octachlorostyrene</th>
<th>total PCBs</th>
<th>Hexa Chloro benzene</th>
<th>trans-Chlorodane</th>
<th>p,p'-DDE</th>
<th>p,p'-DDT</th>
<th>Methyl Triclosan</th>
<th>gamma-BHC (Lindane)</th>
<th>Total PBDE</th>
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<tbody>
<tr>
<td>LVB</td>
<td>3.30</td>
<td>94</td>
<td>0.49</td>
<td>1.59</td>
<td>90.7</td>
<td>3.0</td>
<td>18.5</td>
<td>2.32</td>
<td>118.80</td>
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<tr>
<td>WB</td>
<td>0.45</td>
<td>381</td>
<td>0.27</td>
<td>1.06</td>
<td>44.6</td>
<td>0.9</td>
<td>1.9</td>
<td>0.61</td>
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<tr>
<td>OA</td>
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<td>16</td>
<td>.</td>
<td>0.42</td>
<td>19.2</td>
<td>0.6</td>
<td>0.30</td>
<td>0.05</td>
<td>21.1</td>
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<td>GREGG</td>
<td>.</td>
<td>14</td>
<td>.</td>
<td>0.46</td>
<td>10.0</td>
<td>0.1</td>
<td>0.07</td>
<td>0.03</td>
<td>6.4</td>
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</tbody>
</table>

*Kathy Echols, CERC, USGS*
Willow Beach National Fish Hatchery
Spermatogenesis/Staging
Summary

Protocols for shipping, handling, and sperm quality analyses have been verified and standardized for carp and razorbacks.

Useful for biomonitoring and genetic conservation.

Multiple biomarkers and species, over more than one year provides biologically relevant information.

Male gamete quality differences exist among sites.
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