A proteomics strategy for protein expression profiling and biomarker discovery in wildlife: effects of endocrine disrupting chemicals in frog (Xenopus laevis)

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Proteomics = the study of all proteins expressed by the genome of a given cell or tissue of an organism.
EASYRING biomarker discovery strategy

(EASYRING - Environmental Agent Susceptibility Assessment Utilizing Existing and Novel Biomarkers as Rapid Non Invasive Testing Methods) - EU FP5 project associated with the CREDO cluster (2003-2005)

Test species
- Common carp (Cyprinus carpio)
- African clawed frog (Xenopus laevis)

Aquaria/ cell culture exposures
- EE2
- MDHT
- TAM
- FLU
- + Lambro river water (Italy)

Sampling
- Mucus
- Liver
- Plasma
- Hepatocytes
- MVLN cells
- Culture medium

Ab production and assay development
Biomarker candidates
2-DE/MS
Carp and Xenopus exposures

Carp: 4 doses, 2 weeks, continuous
Xenopus: 1 dose ($10^{-8}$M), 4 weeks, semi-static
Mini 2-DE of X. laevis plasma

Vitellogenin Serotransferrin precursor

Control

10^-8 M MDHT

10^-8 M EE2

Complement C3 Actin

Immunoglobulin light chain

Serotransferrin precursor

Albumin

Estrogen regulated protein Ep45

Unidentified

5 µg protein, IPG pH 3-10 NL

250 kDa
150 kDa
100 kDa
75 kDa
37 kDa
25 kDa
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<th>Accession</th>
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59 MGC0785 protein (Aldehyde dehydrogenase 2 family)
   MGC0785 protein (Aldehyde dehydroge
genase 2 family)
   Acn, cytoplasmic type 5
   Acrin. Aminoacylase 1
   GDP disaccharide inhibitor 2
   Hypothetical LOC49713 (Locate dehydrogenase 1)
   Hypothetical LOC49713 (Locate dehydrogenase 1)
   Mixture of Argininosuccinate synthase and
   E lương factor 1 gamma
   Argininosuccinate synthase 1
   Senesence marker protein-30
   Mixture of Fructose-1,6-biphosphatase and
   Phosphofructokinase related
   MGC2518 protein (Ermr/cadherin/mesoin family)
   Arginase
   Fructose-1,6-biphosphatase
   Hypothetical protein MGC854664 (3-hydroxysterolamine 3-
diolsynthase)
   Hypothetical protein MGC856844 (3-hydroxysterolamine 3-
diolsynthase)
   Glycerol kinase
   Hypothetical protein MGC35995 (RNA pol II accessory
   factor, Cdc75 family) and
   Hypothetical protein MGC83218 (Ribose 5-phosphate
   isomerase)
   L-lactate dehydrogenase B chain
   L-lactate dehydrogenase B chain
   L-lactate dehydrogenase B chain
   Lactate dehydroge
genase A and
   Unknown (protein for MGC11513) (Methylene basic
   protein)
   Lactate dehydrogene
   A
   Unknown (protein for DAGE-481355) (Tetraspecipptide repeat domain)
   Phosphoglucomutase 1
   Heat shock protein gp96 (Hsp90 family)
   Glucose regulated protein, 58 kDa (Protein disulfide
   isomerase)
   Aminoacylase 1 and
   Translation initiation factor IF2A II
   Unknown (protein for DAGE-513341) (Argynin-
   RNA synthase)
   Fumarylacetoacetate hydrolase
   Glutamate dehydrogenase 1
   MGC38368 protein (Phosphoethealaneamine N-
methyltransferase) or
   Mixture of MGC9608 protein (Protein disulfide
   isomerase, A, P5 subfamily) and
   Keratin 18
   Keratin 6 and
   Protein disulfide isomerase-related protein (Thioredoxin
   domain)
   MGC79098 protein (Protein disulfide isomerase)
   Fibronectin gamma chain precursor
   LOC495086 protein (ATP citrate lyase)
   Similar to ubiquitin-cytoskeleton c reductase core
   protein 1
   A2Z (PDI-type ATP synthase, alpha subunit)
   Unknown (protein for MGC-52648) (Hsp 70 family
   Unknown (protein for MGC-52648) (Hsp 70 family
   Glycyl-tRNA synthetase
   Transketolase

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AA577908 577277.55 192 8.8e-16 42 68(23)
AA577908 577277.55 164 5.8e-33 51 100(27)
AA577819 444035.43 74 0.0006 35 95(13)
AA578017 509675.44 91 1.2e-05 50 100(17)
AA82651 472195.77 150 1.4e-11 53 81(21)
AA82651 472195.77 161 1.1e-12 54 74(23)
AA54941 471197.57 142 8.9e-11 36(18)
CA644567 501017.55 - - - -
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BA93719 334515.18 166 3.5e-13 68 100(24)
AA53784 371855.78 233 7e-20 - 100(36)
AA56120 391265.68 - - - -
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AA45237 480575.32 85 4.4e-05 37 87(15)
AA97633 749913.31 186 3.5e-15 40 63(28)
AA54283 489786.22 63 0.0075 36 87(14)
AA77910 595288.03 64 0.0061 31 100(18)
AA78119 572785.25 59 0.019 22 66(10)
AA77228 481535.11 186 3.5e-15 - 97(34)
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AA48887 422855.01 125 4.4e-09 30 37(13)
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AA41200 724895.03 120 1.6e-06 36 96(22)
AA41200 724895.03 135 4.4e-10 40 86(22)
AA77232 849576.98 92 9.9e-06 31 95(21)
AA56101 683786.27 80 0.00016 30 75(24)
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a) Abbreviations used: #, number; Theor., theoretical; Seq. Cov. (%), percent sequence coverage.

b) Protein identified with a peptide tolerance of 150 ppm. Unless otherwise stated all other proteins were identified using a peptide tolerance of 100 ppm.

c) *, Information on protein name/family was obtained by homology searches using Blastp.

d) Protein scores greater than 54 were significant (p<0.05).
Proteins identified in X. laevis plasma

- 123 identified proteins
- Constitutive + differentially regulated
- 19 protein families

- Albumin
- Endodermin
- Alpha-2-macroglobulin
- Apolipoprotein A1
- Complement C3/C4
- Enolase 3
- Creatine kinase
- Estrogen regulated proteinEp45 precursor
- Immunoglobulin heavy and light chain
- Fibrinogen (alpha, beta, gamma)
- Fructose-1,6-bisphosphate aldolase
- Actin
- Serotransferrin precursor
- Triosephosphate isomerase
- Vimentin
- Ficolin-1
- Fetuin
- Hypotetical proteins

Control, pooled male plasma, 500 ug, CBB-G250

Differentially regulated proteins
Differential expression in X. laevis liver

Male, 10^{-8} M EE2, pooled/individual, $\geq$ 2-fold regulation

Complex pattern of expression

430 proteins differentially regulated by EDC treatment

106 differentially regulated spots represented identified proteins
Proteins identified in X. laevis liver

241 analyzed proteins
196 identities
131 unique protein spots
17% of the protein spots contained > 1 protein

67 kDa laminin receptor precursor
ATP synthase alpha/beta
Tubulin, beta 5 and 7 alpha
Keratin
Thioredoxin
Catalase
DEAD box helicase
GDP dissociation factor
Glutaminyl t-RNA synthetase
Fibrinogen gamma chain precursor
Insulinase

10^{-8} \text{ M } \text{EE2, female, 500 µg pooled liver}

- Carbamoyl phosphate synthetase
- Hsp 90b
- Hsp 70
- Calreticulin
- Thioredoxin
- p97 subunit of 15S Mg(2+) ATPase
- Serum albumin B precursor
- Glycyl t-RNA synthetase
- Catalase
- Aldehyde dehydrogenase
- Argininosuccinate synthase
- Fructose-1,6-bisphosphatase
- Vitellogenin
- Ribosomal protein L-10
- Fructose-1,6-bisphosphate aldolase/7-keto-8-aminopelargonate synthetase
Proteins identified in X. laevis liver

241 analyzed proteins
196 identities
131 unique protein spots
17% of the protein spots contained > 1 protein

67 kDa laminin receptor precursor
ATP synthase alpha/beta
Tubulin, beta 5 and 7 alpha
Keratin
Thioredoxin
Catalase
DEAD box helicase
GDP dissociation factor
Glutaminyl t-RNA synthetase
Fibrinogen gamma chain precursor
Insulinase

10^-8 M EE2, female, 500 µg pooled liver

Carbamoyl phosphate synthetase
Hsp 90b
p97 subunit of 15S Mg(2+) ATPase
Serum albumin B precursor
Glycyl t-RNA synthetase
Hsp 70
Calreticulin
Thioredoxin
Fructose-1,6-bisphosphatase
Ribosomal protein L-10
Fructose-1,6-bisphosphate aldolase/7-keto-8-aminopelargonate synthetase
Vitellogenin
Catalase
Aldehyde dehydrogenase
Argininosuccinate synthase
Gene ontology (GO), is a controlled vocabulary used to describe molecular functions, biological processes and the location of gene products.
1-D SDS-PAGE and Vtg-western blot of plasma

**Female**

<table>
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<th>kDa</th>
<th>Control</th>
<th>EE2, 10^{-8} M</th>
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<td>37</td>
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<tr>
<td>25</td>
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</tbody>
</table>

7.5% PA

**Male**

<table>
<thead>
<tr>
<th>kDa</th>
<th>Control</th>
<th>EE2, 10^{-8} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td></td>
<td></td>
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<tr>
<td>150</td>
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</tbody>
</table>

7.5% PA

Anti-xenopus Vtg 1:2000

Vtg
Development of dipstick for endocrine disruption monitoring - non-disruptive sampling of fish mucus
Detection of Vtg in carp mucus

Development and testing of Vtg LFIA

LFIA = Lateral Flow Immunoassay
Rapid non-invasive testing method for detection of Vtg in carp mucus.
Vtg in plasma vs. mucus
Vtg in plasma vs. mucus
Vtg in plasma vs. mucus
Vtg in plasma vs. mucus

EE2, MDHT, FLU
Vtg in plasma vs. mucus

EE2, MDHT, FLU
Vtg in plasma vs. mucus

EE2, MDHT, FLU

TAM  Field
Vtg in carp plasma vs. mucus

Oneway Analysis of log ELISA plasma By Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma Vtg µg/ml ±SD (N)</th>
<th>Mucus Vtg µg/ml ±SD (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>202±310 (10)</td>
<td>0.41±0.32 (8)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>86±99 (10)</td>
<td>0.54±0.97 (9)</td>
</tr>
<tr>
<td>EE2 – 1 ng/l</td>
<td>169±157 (10)</td>
<td>1.04±1.93 (9)</td>
</tr>
<tr>
<td>EE2 – 4 ng/l</td>
<td>775±1278 (9)</td>
<td>5.25±6.11 (9)*</td>
</tr>
<tr>
<td>EE2 – 16 ng/l</td>
<td>5935±4453 (8)*</td>
<td>27.6±11.6 (7)*</td>
</tr>
<tr>
<td>EE2 – 64 ng/l</td>
<td>35355±20978 (10)*</td>
<td>30.8±25.5 (2)*</td>
</tr>
</tbody>
</table>

* Significantly different from untreated control, Dunnetts test of log-transformed data (p<0.05)

Carp Vtg ELISA kit (Biosense)
Estrogen regulated protein Ep45 precursor (Ep45)

- Belongs to the serpin superfamily of proteinase inhibitors
- Similarity to Hu alpha-1-antitrypsin, the major plasma serpin
- Absent in control, 6-fold increase in expression within 8 days by E2 exposure
- Induction parallels that of Vtg
- Possible role in female reproduction by protecting Vtg from proteolytic cleavage during transport

Xenopus plasma: Anti-Ep45-peptide western blots

Xenopus laevis plasma; females exposed to EE₂ / controls - EP45 detection.

controls
p-b1 p-b4 p-b8 p-b12 p-b16
250 →
150 →
100 →
75 →
50 →
37 →

Xenopus laevis plasma; females exposed to Flu / MDHT EP45 detection.

EE2
FLU
MDHT
TAM
Lambro
p-b12 p-b15 p-b16
p-b18 p-b22 p-b23 p-b24 p-b28 p-b37 p-b38 p-b39
250 →
150 →
100 →
75 →
50 →
37 →

Xenopus laevis plasma; males exposed to EE₂ / controls - EP45 detection.

controls
p-b47 p-b51 p-b55 p-b59 p-b63
250 →
150 →
100 →
75 →
50 →
37 →

Xenopus laevis plasma; males exposed to Flu / MDHT EP45 detection.

EE2
FLU
MDHT
TAM
Lambro
p-b2 p-b4 p-b7 p-b10 p-b13 p-b14
p-b17 p-b19 p-b20 p-b21 p-b33 p-b36 p-b35 p-b40
250 →
150 →
100 →
75 →
50 →
37 →
Xenopus plasma: Anti-Ep45-peptide western blots

Xenopus laevis plasma; females exposed to EE₂ / controls - EP45 detection.

Xenopus laevis plasma; males exposed to EE₂ / controls - EP45 detection.

Xenopus laevis plasma; females exposed to Flu / MDHT EP45 detection.

Xenopus laevis plasma; males exposed to Flu / MDHT EP45 detection.

Xenopus laevis plasma; females exposed to TAM / Lambro EP45 detection.

Xenopus laevis plasma; males exposed to TAM / Lambro EP45 detection.
Fig. 3. Vitellogenin (VTG) plasma protein levels of male and female *X. laevis* according to Figs. 1 and 2. The concentration is given as μg/mL. Significant deviations from the control were tested by one-way ANOVA, Dunnett’s-test and are indicated by asterisks (** = p < 0.01, * = p < 0.05).
Xenopus plasma: Anti-serotransferrin-peptide western blots

Xenopus laevis plasma; females exposed to EE2 / controls - Serotransferrin detection.

- EE2 controls p-b41 p-b44 p-b45 p-b46 p-b26 p-b27 p-b28 p-b32
- FLU p-b1 p-b5 p-b8 p-b12 p-b15 p-b16
- MDHT p-b31 p-b32 p-b33 p-b34 p-b35 p-b36
- TAM p-b16 p-b22 p-b23 p-b24 p-b36 p-b37 p-b38 p-b39

Xenopus laevis plasma; males exposed to EE2 / controls - Serotransferrin detection.

- EE2 controls p-b47 p-b48 p-b25 p-b29 p-b30 p-b31
- FLU p-b2 p-b3 p-b4 p-b7 p-b10 p-b11 p-b13 p-b14
- MDHT p-b17 p-b19 p-b20 p-b21 p-b23 p-b34 p-b35 p-b40

Xenopus laevis plasma; females exposed to Flu / MDHT Serotransferrin detection.

- TAM / Lambro
Xenopus plasma: Anti-serotransferrin-peptide western blots

Xenopus laevis plasma; females exposed to EE2 / controls - Serotransferrin detection.

Xenopus laevis plasma; females exposed to Flu / MDHT - Serotransferrin detection.

Xenopus laevis plasma; males exposed to EE2 / controls - Serotransferrin detection.

Xenopus laevis plasma; males exposed to Flu / MDHT - Serotransferrin detection.
Xenopus plasma: Anti-fibrinogen β -peptide western blots

**Xenopus laevis plasma; females exposed to EE₂ / controls - fibrinogen beta detection.**

**Xenopus laevis plasma; females exposed to Flu / MDHT fibrinogen beta detection.**

**Xenopus laevis plasma; females exposed to TAM / Lambro - fibrinogen beta detection.**
Xenopus plasma: Anti-fibrinogen β-peptide western blots

Xenopus laevis plasma; females exposed to EE2 / controls - fibrinogen beta detection.

<table>
<thead>
<tr>
<th>Controls</th>
<th>EE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-b41</td>
<td>p-b44</td>
</tr>
<tr>
<td>p-b45</td>
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</tr>
<tr>
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<td>p-b32</td>
<td>p-b35</td>
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250 →
150 →
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Xenopus laevis plasma; females exposed to Flu / MDHT fibrinogen beta detection.

<table>
<thead>
<tr>
<th>FLU</th>
<th>MDHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-b1</td>
<td>p-b5</td>
</tr>
<tr>
<td>p-b8</td>
<td>p-b9</td>
</tr>
<tr>
<td>p-b12</td>
<td>p-b15</td>
</tr>
<tr>
<td>p-b16</td>
<td></td>
</tr>
</tbody>
</table>

250 →
150 →
100 →
75 →
50 →
37 →

Xenopus laevis plasma; females exposed to TAM / Lambro - fibrinogen beta detection.

<table>
<thead>
<tr>
<th>TAM</th>
<th>Lambro</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-b18</td>
<td>p-b22</td>
</tr>
<tr>
<td>p-b23</td>
<td>p-b24</td>
</tr>
<tr>
<td>p-b36</td>
<td>p-b37</td>
</tr>
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<td>p-b38</td>
<td>p-b39</td>
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</tbody>
</table>

250 →
150 →
100 →
75 →
50 →
37 →

Xenopus laevis plasma; males exposed to EE2 / controls - fibrinogen beta detection.

<table>
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<th>Controls</th>
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<tr>
<td>p-b47</td>
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250 →
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Xenopus laevis plasma; males exposed to Flu / MDHT fibrinogen beta detection.

<table>
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<tbody>
<tr>
<td>p-b2</td>
<td>p-b3</td>
</tr>
<tr>
<td>p-b4</td>
<td>p-b7</td>
</tr>
<tr>
<td>p-b10</td>
<td>p-b11</td>
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<tr>
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250 →
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Xenopus laevis plasma; males exposed to TAM / Lambro - fibrinogen beta detection.

<table>
<thead>
<tr>
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<tr>
<td>p-b17</td>
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250 →
150 →
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Xenopus plasma: Anti-fibrinogen β-peptide western blots

Xenopus laevis plasma; females exposed to EE2/controls - fibrinogen beta detection.

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Xenopus laevis plasma; males exposed to TAM / Lambro - fibrinogen beta detection.
Proteome changes in Atlantic cod larvae exposed to produced water
Biomarker discovery in Atlantic cod fry liver after continuous exposure to produced water

Kjersem (2007), PhD thesis
Figure 1  Process flow for the development of novel protein biomarker candidates. ‘Numbers of analytes’ refers to the number of proteins expected to be evaluated as candidate biomarkers in each phase of development. ‘Numbers of samples’ refers to the sample requirements for each phase. LC-MS/MS, liquid chromatography tandem mass spectrometry; SID, stable isotope dilution; MRM, multiple reaction monitoring.
Conclusions
Conclusions

A toxicoproteomic strategy has been established to identify biomarker candidates under various exposure regimes in different species.

Higher identification success rates are obtained in species with better genomic coverage (e.g. X. laevis > carp > cod).

Proteome changes linked to annotated databases and Gene Ontology terms may help elucidates toxicological mechanisms and modes of action.

In general, responses specific to a single chemical are interesting as biomarker candidates, however suites of biomarkers may prove more informative in field studies targeting emerging pollutants - e.g. applied in protein/antibody arrays.
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EASYRING partners (2003-2005):
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Anne Van Cauwenberge, UMH, Mons, Belgium
Mark Cronin, LJMU, Liverpool, UK

The Institute of Marine Research, Bergen, Norway
Computational Biology Unit, University of Bergen

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