Form 3

Annual Report

LaMer, Ehime University

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To Director of LaMer

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Include the report on the result of the project/meeting in a separate sheet.

1. Project / Meeting title

iTRAQ-based proteome response of cane toads (*Rhinella marina*) exposed to chemical pollutants in the wild

2. Members of project / meeting

Name	Affiliation	Position	Contribution part
PI: Maria Claret Tsuchiya	University of the Philippines Los Banos	Assistant Professor	Research concept and experimental design, Conduct of experiment, Data analysis, Writing of report
Members: Jashin Rosal	University of the Philippines Los Banos	Graduate Student (M2)	Conduct of experiment, Data analysis, Writing of report
LaMer Faculty member in charge: Hisato Iwata	CMES, Ehime University	Professor	Research concept and experimental design, Data analysis, Writing of report

AIM

In this study, application of iTRAQ labeling quantitative proteomic technology for global characterization of the toad liver proteome exposed in the contaminated and reference sites were used. Specifically, this study aimed to detect the changes of protein expression in cane toads in response to contaminants.

PROCEDURE

Sample collection Cane toads were collected from February 22 – March 4, 2013 in Sta. Rosa and Los Banos, Laguna, and in Bilar, Bohol, Philippines. The animals were immediately dissected on board after measurement of biometry (body length, body weight, etc.). Organ (liver, spleen, muscle, and kidney) samples were removed and total organ weight was measured. The subsamples were frozen in liquid nitrogen, transported to Ehime University, Japan, and stored at -80°C.

Preparation of total protein fraction Liver tissue (~100 mg) were homogenized in five volumes of cold lysis buffer (0.1M Tris HCl, pH 7.8, 0.05M DTT, 1x complete mini protease cocktail). 20% SDS (final concentration 2%) was added to the homogenate and sonicated 3 times at 10% x 5 cycles for 30 sec. The samples were incubated at 95°C for 5 min and cooled down at RT prior to centrifugation at 16000g for 15 min. The supernatant containing the total protein was collected and stored at -80°C until further analysis.

Preparation of microsomal fraction Microsomes were prepared as reported by Hirakawa et al (2007). Tissue (~6 g) from liver was homogenized in five volumes of cold homogenization buffer (50 M Tris-HCl, 0.15M KCl, pH 7.4–7.5) with a teflon-glass homogenizer (10 passes), and centrifuged for 10 min at 750 × g. The supernatant will then be centrifuged at 12,000 × g for 10 min. The supernate was further centrifuged at 105,000 × g for 70 min. The supernatant (cytosol) fraction was removed, and microsomal pellets was resuspended in one volume of resuspension buffer [50mM Tris-HCl, 1mM EDTA, 1mM dithiothreitol, 20% (vol/vol) glycerol, pH 7.4–7.5]. All the microsomal fractions were immediately frozen in liquid nitrogen, and stored at -80°C until further CYP enzyme assays and immunoblotting.

Protein concentrations were determined by the bicinchoninic acid method (Smith et al., 1985). BCA Protein Assay Reagent (Pierce, Rockford, IL) was used for the protein assay and bovine serum albumin as a standard. Absorbance at 560 nm was measured using a multiwell plate reader (SpectraFluor Plus, Tecan Austria GmbH, Groedig, Austria).

Protein Digestion, iTRAQ® labeling, and Fractionation Protein solubilization and trypsin digestion were done using FASP Protein Digestion kit (Expedeon). Briefly, 100µg total protein lysate was mixed with urea sample solution in the spin filter and centrifuged at 14000g for 15 min. IAA solution was added, incubated in the dark at RT for 20 min, and centrifuged. The spin filter was added with urea sample and centrifuged. 75µl of digestion solution containing trypsin (enzyme to protein ratio 1:100) was added and incubated overnight with shaking at 37°C. 50mM ammonium bicarbonate was added to elute the digested peptides. The peptide mixture was desalted, and mixed with iTRAQ® reagents for 2h at RT. All labeled peptides were combined and fractionated using iCAT® cation exchange chromatograph.

Protein Identification by nano-LC-MALDI TOF/TOF MS and Homology search The labeled peptide mixture was desalted using C18 Spin Tips (Thermo Fisher Scientific, USA) and resuspended in 2% ACN, 0.1% TFA. 20ul was injected to the nano-LC for elution into MALDI sample plate with the matrix solution (10 mg/mL α-cyano-carboxycinnamic acid in 50% ACN/0.1% TFA) and then analysed on MALDI TOF/TOF mass spectrometer (5800 Plus MALDI TOF/TOF™, AbSciex). Peptide identification was analyzed using the Protein Pilot software against the Uniprot *Xenopus* database.

Western Blotting

Equal amount microsomal proteins of each sample were loaded onto precast 4%-12% Bis-Tris gels (Invitrogen) and ran in MOPS buffer under reduced conditions. Electrophoresed proteins were then transferred to a nitrocellulose membrane using iBlot (Invitrogen) according to the standard protocol. The membranes were blocked with TBS-T containing 5% nonfat milk powder and 0.1% Tween 20 at 4°C overnight. CYP1A1 and CYP1B1 isoforms were detected using rabbit polyclonal anti-CYP1A1 (1:1000, Sta. Cruz Biotechnology), and rabbit polyclonal anti-CYP2B (1:1000, Sta. Cruz Biotechnology, respectively. Appropriate secondary antibody conjugated with horseradish peroxidase (HRP) was used to visualize the primary immune-recognitions using the ECL kit (ECL, Amersham Pharmacia Biotech) and imaging analyzer ChemiDoc (Bio-Rad).

RESULTS

Total Protein and Microsome Content

The total protein content and microsome content was measured using the BCA assay. Table 1 and Figure 1 show the total protein and microsome concentration of the liver samples obtained from the three sampling sites. The sampling site that obtained the highest concentration of hepatic total proteins was Bohol (23.50 mg/ml) while Los Baños obtained the highest concentration of microsomal proteins with an average of 12.64 mg/ml. One-way analysis of variance showed that the total proteins from the three sampling sites were statistically significant (*P*=0.001). Total protein samples from Los Baños had a 0.7x fold-decrease than Bohol (*P*=0.001) while Sta. Rosa had a 0.8x fold-decrease than Bohol (*P*=0.02). On the other hand, microsome content among the three sampling sites were not statistically significant (*P*=0.114). There was a 1.2x fold increase of the average microsome concentration in Los Baños compared to Bohol while no increase on the average microsome content in Sta. Rosa with reference to the data from Bohol.

Table 1. Total protein and microsome concentrations in Cane toad liver from three sampling sites in the Philippines.

Content	Los Baños	Sta. Rosa	Bohol
Total Protein (mg/ml)	17.32 ±1.29	19.28 ±0.60	23.50 ±1.19
Microsome (mg/ml)	12.62 ±1.01	10.57 ±0.68	10.57 ±0.54

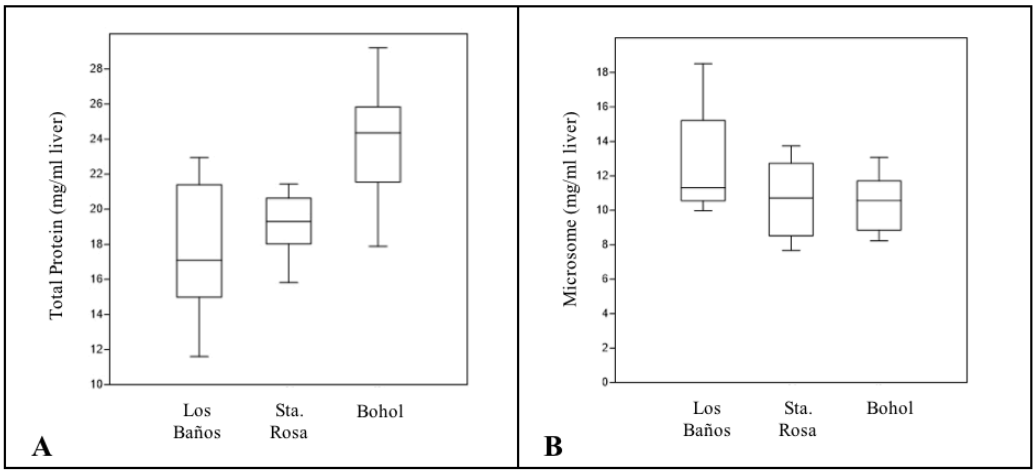

Figure 1. Total protein (A) and microsome (B) concentration measured from Cane toad liver samples obtained from the three sampling sites in the Philippines.

Toad Liver Proteome

All MS/MS spectra were processed using the ProteinPilot sd searched against the *Xenopus* UNIPROT Database. To determine the differentially expressed proteins, the 1.5-fold increase or decrease in protein expression was used as benchmark for significant change. For the uncharacterized proteins, the BLASTp of the NCBI database was used against all databases. Proteins were categorized based on their molecular functions—binding, catalytic activity, structural molecule activity, and transporter activity.

Los Baños, Laguna Site

There were a total of 62 differentially expressed proteins (DEPs) from the female cane toads from Los Baños, Laguna. 31 proteins were upregulated and 31 proteins were down-regulated. GO search of these DEPs belong to 26 molecular functions.

Nucleic acid binding proteins were up-regulated and these are the uncharacterized protein F6Q732 (1.76-fold), histone H2B *(LOC108648865)* (1.66-fold), and helicase with zinc finger (2.30-fold).

Downregulated proteins under the protein binding category include microtubule-actin crosslinking factor 1 (0.46-fold), CREB-binding protein (0.42-fold), guanine nucleotide binding protein (G protein), beta polypeptide (0.64-fold), and myosin IC (0.67) while the reticulon 4 receptor (1.52-fold) was up-regulated. For the chromatin binding, the CREB-binding protein (0.42-fold) was down-regulated.

Regarding proteins with oxidoreductase activity, cytochrome P450 4B1-like (1.87-fold) was up-regulated, while homogentisate 1,2-dioxygenase (0.66-fold) was down-regulated. For transferases, an uncharacterized protein F6Q732 (1.76-fold) and the betaine--homocysteine S-methyltransferase 1 (1.51-fold) were both up-regulated, and CREB-binding protein (0.42-fold) was down-regulated.

Only one down-regulated protein has a signal transducer activity, guanine nucleotide binding protein (G protein), beta polypeptide (0.65-fold). This protein is involved notably on angiogenesis, regulation of cell division, and protein localization. Enzyme regulators myosin IC (0.67-fold) PKHD1, fibrocystin/polyductin (0.46-fold) was down-regulated while helicase with zinc finger (2.30-fold) was up-regulated.

Meanwhile, three genes were involved in the transporter activity, specifically on the transmembrane transporter activity. Anion exchange protein slc4a7 (1.60-fold) and voltage-dependent T-type calcium channel subunit alpha cacna1h (1.66-fold) were up-regulated, while amino acid transporter (0.62-fold) was down-regulated.

All proteins under the structural molecule activity were down-regulated. This includes the structural component of cytoskeleton, microtubule-actin crosslinking factor 1 (0.46-fold) and myosin IC (0.47-fold), and structural constituent of ribosomes, ribosomal protein S15 (0.26-fold) and LOC100170552 protein *(rpl27a)* (0.43-fold).

Sta. Rosa, Laguna Site

In the male cane toads from Sta. Rosa, Laguna, 60 proteins were differentially expressed: 26 up-regulated proteins and 34 down-regulated proteins. On the other hand, female toads showed 83 differentially expressed proteins which include 37 up-regulated proteins and 46 down-regulated proteins.

Proteins with binding activity can be subdivided into four categories—chromatin binding, nucleic acid binding, nucleoside binding, and protein binding. Chromatin binding include ATPase family, aaa domain-containing 2B (1.56-fold), CREB-binding protein (0.44-fold) and ATPase family, AAA domain-containing 2B (0.49-fold). For the nucleic acid binding, up-regulated proteins were zinc finger and btb domain-containing protein 17 F6Q732, E3 ubiquitin-protein ligase RBBP6 F6T9J9 (1.85-fold), ribosomal protein S14 (1.57-fold), histone H2A h2afj (1.57-fold), histone H2B LOC100486870 ortholog (1.57-fold), and helicase with zinc finger domain-related (2.60-fold). Down-regulated proteins were core histone macro-H2A (0.59-fold), CREB-binding protein (0.50-fold), uncharacterized protein *(cyfip2)* (0.23-fold), uncharacterized protein *(rapgef6)* (0.58-fold), ring finger protein 112, gene 2 (0.60-fold), dynein axonemal heavy chain 14 (0.65-fold), microtubule-actin crosslinking factor 1 (0.53-fold), CREB-binding protein (0.50-fold), immunoglobulin superfamily member 10 (0.65-fold), Dystonin (0.54-fold), ATPase family, AAA domain-containing 2B (0.49-fold), Novel Rho protein (0.61-fold), and Myosin IC (0.46-fold). The nucleotide binding was solely represented by novel rho protein (0.61-fold). This protein has a biological role in wound healing, GTPase mediated signal transduction, and skeletal muscle satellite cell migration. For protein binding, up-regulated were Reticulon 4 receptor (1.79-fold), ATPase family, AAA domain-containing 2B (1.56-fold), Fibrinogen alpha chain (1.50-fold), and Guanylate-binding protein 7 (1.67-fold); uncharacterized protein *(wdr63)* (0.61-fold) was down-regulated.

Proteins with catalytic activity can be categorized into seven sub-groups: enzyme regulator activity, helicase activity, hydrolase activity, isomerase activity, lyase activity, oxidoreductase activity, and transferase activity. All proteins under the enzyme regulator activity were down-regulated including uncharacterized protein *(rapgef6)* (0.59-fold) and Myosin IC (0.58-fold). Helicase activity was solely represented with an up-regulated protein Helicase with zinc finger (2.58-fold). Under the hydrolase activity, up-regulated proteins were RB binding protein 6, ubiquitin ligase (1.54-fold), ATPase family, AAA domain-containing 2B (1.56-fold), lactase gene 2 (1.71-fold), SAM domain and HD domain 1 (1.63-fold), and carboxypeptidase (1.62-fold). While down-regulated proteins were uncharacterized protein *(wdr63)* (0.61-fold), ring finger protein 112, gene 2 (0.60-fold), dynein axonemal heavy chain 14

(0.65-fold), PKHD1, fibrocystin/polyductin (0.56-fold), V-type proton ATPase proteolipid subunit (0.62-fold), eyes absent homolog (0.47-fold), novel Rho protein (0.61-fold), and myosin IC (0.58-fold). All lyases were downregulated; these include the uncharacterized protein *(pck2)* (0.62-fold), uncharacterized protein *(cryl1)* (0.49-fold), cysteine sulfinic acid decarboxylase (0.62-fold), and 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1) (0.62-fold).

Cytochrome P450 4B1-like *(LOC100488227)* (1.67-fold) was up-regulated. But other oxidoreductases like apoptosis-inducing factor, mitochondria-associated 1 (0.58-fold) and uncharacterized protein *(cryl1)* (0.49-fold) were down-regulated. For those with transferase activity, up-regulated proteins were uncharacterized protein (fragment) (1.69-fold), transformation/transcription domain-associated protein (1.57-fold), and alanine aminotransferase 2 (1.57-fold), while the ketohexokinase (0.64-fold), CREB-binding protein (0.50-fold), Pim-3 proto-oncogene, serine/threonine kinase (0.66-fold), and UDP glucuronosyltransferase 2 family, polypeptide A1, complex locus (0.64-fold) were down-regulated.

An uncharacterized protein F6Z4K8 (0.56-fold) with receptor activity was down-regulated. The signal transducer activity was represented by another uncharacterized protein F6Z4K8 (0.56-fold) and novel Rho protein (0.61-fold), both of which are also down-regulated.

Several structural molecules mostly down-regulated and these include the microtubule-actin crosslinking factor 1 (0.53-fold), collagen type IV, alpha 2 (0.45-fold), dystonin (0.55-fold), actin alpha 2, smooth muscle, aorta (0.63-fold), and myosin IC (0.58-fold) which were down-regulated. Further, except the uncharacterized protein F7EAQ4 (1.52-fold), all proteins with structural activity were down-regulated. These include the amino acid transporter (Fragment) (0.46-fold), V-type proton ATPase proteolipid subunit (0.62-fold), and polycystin 2, transient receptor potential cation channel (0.48-fold).

Bohol Site

There were a total of 90 differentially expressed proteins in the male cane toads from Bohol site. Of these, 42 proteins were up-regulated and 48 proteins were down regulated. Female cane toads showed higher number (112) of differentially expressed proteins that include 52 up-regulated and 60 down-regulated proteins.

Some proteins with chromatin binding activity were down-regulated. Among these are the CREB-binding protein (0.51) and HECT and RLD domain-containing E3 ubiquitin protein ligase 2 (0.61-fold). Similarly, proteins involved in nucleic acid binding were also downregulated except the helicase with zinc finger (2.73-fold). Proteins that were down-regulated under this category were CREB-binding protein (0.51), ribosomal protein S14 (0.50-fold), HECT and RLD domain-containing E3 ubiquitin protein ligase 2 (0.65-fold), Uncharacterized protein *(cyfip2)* (0.61-fold), and Histone H2A.Z-like (0.62-fold).

For protein binding, downregulated proteins were myosin IE, Uncharacterized protein *(wdr63)* (0.66-fold), an uncharacterized protein *(gbp5)* (0.41-fold), ring finger protein 112, gene 2 (0.47-fold), dynein axonemal heavy chain 14 (0.57-fold), uncharacterized protein *(XENTR_v90028848mg)* (0.59-fold), microtubule-actin crosslinking factor 1 (0.25-fold), CREB-binding protein (0.49-fold), immunoglobulin superfamily member 10 (0.59), guanine nucleotide binding protein (G protein), beta polypeptide (0.61-fold), dystonin (0.51-fold), uncharacterized protein *(kif14)* (0.58-fold), dynein cytoplasmic 1 heavy chain 1 (0.66-fold), galectin (0.52-fold), potassium channel, calcium-activated intermediate/small conductance subfamily N alpha, and myosin IC (0.49-fold). On the other hand, only two proteins under this category were up-regulated: gene 1 (1.95-fold) and member 4 (1.50-fold).

The catalytic activity is subdivided into five categories: (1) ATPase activity coupled to transmembrane movement of substances, (2) GTPase activity, (3) hydrolase activity acting on ester bonds, (4) hydrolase activity hydrolyzing O-glycosyl compounds, and (5) phosphatase activity. The ATPase activity involved two down-regulated proteins, the ATP synthase, H+-transporting, mitochondrial Fo complex subunit G (0.64-fold) and V-type proton ATPase proteolipid subunit (0.59-fold). The GTApase activity includes the down-regulated uncharacterized protein *(gbp5)* (0.41-fold) and Ring finger protein 112, gene 2 (0.49-fold) and down-regulated RAB7A, member RAS oncogene family (2.05-fold). Under the hydrolase activity acting on ester bonds are eyes absent homolog (0.26-fold) and helicase with

zinc finger (2.72-fold). There was a down-regulation of proteins for the hydrolase activity hydrolyzing O-glycosyl compounds and phosphatase activity: PKHD1, fibrocystin/polyductin (0.52-fold) and Eyes absent homolog (0.43-fold), respectively. Guanine nucleotide binding protein (G protein), beta polypeptide (0.61-fold) which is involved in angiogenesis and various developmental processes was down-regulated.

Two up-regulated uncharacterized proteins were found to have receptor activities: uncharacterized protein F6Z4K8 (1.97-fold) and uncharacterized protein *(XENTR_v90029445mg)* (1.59-fold). Meanwhile, three are with signal transducer activity: uncharacterized protein *(map3k15)*(2.05-fold), uncharacterized protein F6Z4K8 (2.32-fold), and Uncharacterized protein *(XENTR_v90029445mg)* (1.59-fold).

There were 10 proteins with structural molecule activity. Nine were down-regulated proteins: ribosomal protein S15 (0.65-fold), uncharacterized protein *(XENTR_v90028848mg)* (0.64-fold), microtubule-actin crosslinking factor 1 (0.40-fold), ribosomal protein S14 (0.50-fold), collagen type IV, alpha 2 (0.41-fold), dystonin (0.49-fold), actin alpha 2, smooth muscle, aorta (0.61-fold), LOC100170552 protein (0.66-fold), and myosin IC (0.48-fold). But myosin IE, gene 1 (1.60-fold), whose function is for ATP and actin binding and motor activity was up-regulated.

Six differentially expressed proteins are with transporter activity. Down-regulated proteins include amino acid transporter (0.31-fold), ATP synthase, H+-transporting, mitochondrial Fo complex subunit G (0.64-fold), V-type proton ATPase proteolipid subunit (0.59-fold), and polycystin 2, transient receptor potential cation channel (0.57-fold). Meanwhile, potassium channel, calcium-activated intermediate/small conductance subfamily N alpha, member 4 (1.84-fold) and an uncharacterized protein *(dennd5a)* (1.63-fold) were up-regulated.

Western Blot Analysis

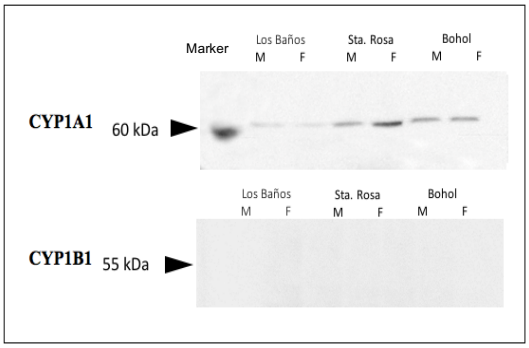

Figure 2. Western blot analysis of CYP1A1 and CYP1B1 in the pooled microsomes (50ug per lane) of liver tissue from *R. marina*.

Cytochrome P-450 monooxegenases are the major compounds in the Phase I system that are known to catalyze the oxidative biotransformation of organic compounds. Among the important members are CYP1A1 and CYP1B1 which are responsible for the metabolism of various drugs, food components, and activates several environmental mutagens, e.g. polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines and aromatic amines.

The expression of CYP1A1 and CYP1B1 in cane toad liver was done by Western blotting and densitometric analysis. CYP1A1 in the male samples from Los Baños was 0.4x fold lower than in Bohol while Sta. Rosa had a 1.3x fold-increase (Figure 2). Female CYP1A1 expression in Los Baños was 0.2x fold lower than Bohol and Sta. Rosa had a 2.2x fold higher than Bohol. Interestingly, CYP1B1 proteins were not expressed in all samples from the three sites.

FUTURE PERSPECTIVES

Further analyses will be done to compare the protein profiles between the male and female toads among the different sites. Oral and poster presentations are expected to be presented from these results. Further studies to confirm the expression of proteins and genes identified are recommended.