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The metamorphosis of amphibian toxicogenomics

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Amphibians are important vertebrates in toxicology often representing both aquatic and terrestrial forms within the life history of the same species. Of the thousands of species, only two have substantial genomics resources: the recently published genome of the Pipid, *Xenopus* (*Silurana*) *tropicalis*, and transcript information (and ongoing genome sequencing project) of *Xenopus laevis*. However, many more species representative of regional ecological niches and life strategies are used in toxicology worldwide. Since *Xenopus* species diverged from the most populous frog family, the Ranidae, ∼200 million years ago, there are notable differences between them and the even more distant Caudates (salamanders) and Caecilians. These differences include genome size, gene composition, and extent of polyploidization. Application of toxicogenomics to amphibians requires the mobilization of resources and expertise to develop *de novo* sequence assemblies and analysis strategies for a broader range of amphibian species. The present mini-review will present the advances in toxicogenomics as pertains to amphibians with particular emphasis upon the development and use of genomic techniques (inclusive of transcriptomics, proteomics, and metabolomics) and the challenges inherent therein.

Keywords: amphibian, frog, molecular techniques, polyploid, endocrine disruptor, estrogen, thyroid hormone, toxicogenomics

Amphibians have an undeniable, yet understated, role in toxicology. They diverged from other vertebrates 360 million years ago (Frost et al., 2006) and, currently, over 60% of the >6,900 known species are threatened or declining in numbers (AmphibiaWeb, 2012). Recent amphibians are comprised of three orders: Anura (frogs and toads), Caudata (salamanders), and Gymnophiona (caecilians; Dubois, 2004; Frost et al., 2006), of which ∼90% of species are Anura. Toxicological studies are primarily on anurans with some studies on caudates. Caecilian representation is lacking. In contrast to the limited ranges of caudate and caecilians, anurans are found on every continent except Antarctica (AmphibiaWeb, 2012).

Amphibians represent the only vertebrate group where a large majority of its members exhibit a life history that includes distinct independent aquatic larval and terrestrial juvenile/adult phases. The transition between the larval and juvenile phases requires substantial or complete remodeling of the organism (metamorphosis) in anticipation of a terrestrial lifestyle. Thus this places amphibians in a unique position for the assessment of toxicological effects in both aquatic and terrestrial environments. Over 10,000 study records on amphibians are currently available on the US Environmental Protection Agency's ECOTOX database (US EPA, 2012). Ninety percent of records represent aquatic exposures and these are biased toward a single species (*Xenopus laevis*). The remaining 10% of records comprise terrestrial exposures of frog, toad, and salamander species (US EPA, 2012). Less than 300 records include any mRNA expression data.

The exquisite sensitivity of frogs to hormones fostered the launching of several initiatives to develop standardized testing methods. Environment Canada is developing a frog tadpole exposure assay that uses native species (*Rana catesbeiana* and *pipiens*) and a combination of molecular and morphological criteria (Veldhoen et al., 2006c). The Organization for Economic Cooperation and Development (OECD, 2009) has recently established guidelines for a standardized assay for evaluating thyroid hormone (TH) active chemicals using a *X. laevis* metamorphosis assay (XEMA) and is in the process of evaluating a multigenerational reproductive assay in *X. tropicalis*. The XEMA assay has been adapted for *X. tropicalis* (Mitsui et al., 2006) and served as a template for the development of some native frog metamorphosis assays for *R. rugosa* (Oka et al., 2009), *Bombina orientalis* (Park et al., 2010), and *Pseudacris regilla* (Marlatt et al., submitted). However, the recommended XEMA assay relies upon morphological criteria and the input of toxicogenomic endpoints is not standard practice. Nevertheless, movement toward inclusion of molecular endpoints to reduce assay time and provide greater information regarding test chemical mode of action is evident in the literature (**Table 1**).

Amphibians are used in two general ways in the context of toxicology: in laboratory exposure settings where individual chemicals or complex mixtures are tested and in the field setting. Although availability of appropriate life stages is year round for some species (e.g., *Xenopus* tadpoles can be bred on demand, *R. catesbeiana* tadpoles can be collected from the wild and housed in aquatics facilities year round), many have limited availability throughout the year. Moreover, field sampling of threatened or endangered species necessitates the development of non-lethal sampling methods (fin biopsies) combined with molecular analyses (Veldhoen and Helbing, 2005). Efforts have also been made to combine transcript analysis with cultured tail fin biopsies

Table 1 | Representative studies using amphibian toxicogenomics.

Approach	Method	Species	Representative publications[a]
Transcriptomics	Microarray	*Rana catesbeiana*	Veldhoen et al. (2006b)
		Xenopus laevis	Helbing et al. (2007), Heimeier et al. (2009), Gohin et al. (2010), Searcy et al. (2012)
	QPCR	*Andrias japonicus*[b]	Katsu et al. (2006)
		Pleurodeles waltl	Ko et al. (2008)
		Pseudacris regilla	Veldhoen et al. (2006a), Marlatt et al. (submitted)
		Rana catesbeiana	Gunderson et al. (2011)
		Rana pipiens	Howe et al. (2004), Duarte-Guterman and Trudeau (2010), Langlois et al. (2010)
		Rana rugosa	Suda et al. (2011)
		Rana temporaria	Mortensen et al. (2006)
		Xenopus tropicalis	Langlois et al. (2011)
		Xenopus laevis	Zhang et al. (2006), Oka et al. (2008), Zimmermann et al. (2008), Baba et al. (2009), Rossi et al. (2009), Massari et al. (2010), Qin et al. (2010), Urbatzka et al. (2010), Zaya et al. (2011)
Proteomics	Two dimensional polyacrylamide gel electrophoresis followed by liquid chromatography and tandem mass spectrometry (2D-PAGE; LC-MS/MS)	*Rana catesbeiana*	Domanski and Helbing (2007), Serrano et al. (2010)
		Xenopus laevis	
	Isobaric tags for relative and absolute quantitation (iTRAQ)	*Rana catesbeiana*	Domanski and Helbing (2007), Serrano et al. (2010)
		Xenopus laevis	
Metabolomics	Ultra performance liquid chromatography Mass Spectrometry (UPLC-MS)	*Rana catesbeiana*	Helbing et al. (2010)
	Inductively coupled plasma mass spectrometry (ICP-MS)	*Xenopus laevis*	Tietge et al. (2010)

[a] *Due to space constraints, the author regrets that all studies could not be included. She has provided selected references representative of key laboratories active in the area and encourages the interested reader to consult the literature for additional published works by the highlighted laboratories.*

[b] *Due to the paucity of publications for salamanders, this work was included in the table even though this study used gel-based quantitation methods for PCR products.*

for rapid screening of chemicals and effluents (Hinther et al., 2010).

Toxicogenomics are best suited for identifying and evaluating factors categorized as sublethal deleterious effects that influence survival and recruitment; the primary factors contributing to amphibian population declines (Hayes et al., 2010). Such factors include: stress, susceptibility to disease, climate change, and environmental pollutants. Typically, molecular responses precede morphological endpoints giving early indications of response and modes of action. To date, application of toxicogenomics to amphibians has been extremely limited due to restrictions in resources/expertise and the difficulty in obtaining consensus on which toxicologically important species to develop large-scale genomics resources for. Approaches for transcriptomics, proteomics, and metabolomics pertaining to frogs has been previously reviewed elsewhere (Helbing et al., 2010). Research efforts have largely focused on the evaluation of endocrine disruption in frogs, primarily with respect to xenoestrogens and TH-active chemicals, including hormonal cross-talk and their complex interactions with environmental factors (**Table 1**). Sex reversal and/or intersex conditions in response to chemical exposures have been reported and some laboratories have begun to examine their molecular basis (**Table 1**). The absolute dependence of frog tadpoles upon TH during metamorphosis into a juvenile frog (Shi, 2000) provides for the most comprehensive and drastic response known to the hormone; although all vertebrates require THs for development, nervous system function, and metabolism (Oppenheimer, 1999). Indeed, the use of frog tadpoles as surrogate species for TH disruption in mammals has been explored at the molecular level (Searcy et al., 2012).

Even where more resources are available (for, e.g., commercially available *Xenopus* oligo microarrays), restrictions in cost and lack of utility across species platforms (Helbing et al., 2010) have greatly limited application of toxicogenomics tools beyond quantitative real time polymerase chain reaction (QPCR; **Table 1**). It is notable that very few relevant studies have been performed using salamanders and none with caecilians (**Table 1**). For labs having the necessary expertise, efforts have concentrated upon the production of transcript-based biomarkers (although some data on proteomics and metabolomics have been published; **Table 1**),

definition of baseline responses to model hormones, identification of appropriate sampling times for molecular biomarker use, and determination of natural variation (**Table 1**).

Most of the toxicogenomic studies that have been published so far are laboratory-based using *X. laevis* strains that are akin to the white mouse of the frog world. *Xenopus* are Pipidae; one of 38 families within the Anura (Frost et al., 2006). Pipidae are distantly related to most Anura and diverged from the largest family, the Ranidae (the true frogs), ∼200 million years ago (Sumida et al., 2004). Marked differences in life histories (for, e.g., *Xenopus* remain aquatic after metamorphosis, whereas Ranids become terrestrial), species sensitivities (Relyea and Jones, 2009), and genome compositions (discussed below) have prompted a call for developing toxicogenomics tools and approaches applicable to environmentally relevant species (Denslow et al., 2007).

Amphibian toxicogenomics has largely been driven through the adoption of tools made available through disciplines outside of toxicology, namely developmental, cell, and molecular biology. This provided, firstly, gene information from *X. laevis*, and, subsequently, a genome from *X. (Silurana) tropicalis*. However, this has not been without difficulty. *X. laevis* has been the most-used amphibian toxicological model due to the ease of husbandry in laboratory settings. However, genetically, *X. laevis* is pseudotetraploid, derived from an ancient tetraploid lineage with incomplete diploidization across a large portion of the genome (Mable et al., 2011). In fact, Pipidae have the highest number of polyploid species identified within amphibians (Mable et al., 2011). This created significant problems in initiating a genome sequencing project for this species due to the bioinformatic challenge of assembling a tetraploid genome *de novo*. Fortunately, a diploid *Xenopus* species did exist with similar husbandry benefits in this family. Thus the sequencing of the first frog genome was performed on *X. tropicalis* and completed in 2010 (Hellsten et al., 2010). The problem for toxicology is that the availability of the *X. tropicalis* genome information is now driving scientists to use this as a test species for use in toxicogenomics. Although an enormous opportunity, it is not clear how suitable this species will be as a representative of native frog species or amphibians in general.

Amphibians present a wide range of species diversity. They contain keystone species within a plethora of ecosystems throughout the world. They are important food sources for humans and wildlife, instrumental in pest control, and serve as sensitive indicators within a variety of ecosystems. With the exception of *R. catesbeiana* which is distributed worldwide, amphibians tend to have regional representation. Therefore, toxicological evaluations have often tended toward regionalism as well [for example, common frog ecotox species are *R. rugosa* (Japan), *R. temporaria* (Europe), and *R. pipiens* (North America)]. Moreover, amphibians have representatives of different sex determination systems (e.g., XX/XY, ZW/ZZ; Eggert, 2004) that could influence sensitivity to environmental contaminants. Genome organization is similar within amphibian subgroups, but varies substantially between subgroups. In addition to polyploidy in a few amphibian species, the genome sizes of amphibians span four orders of magnitude from one-quarter of the human genome (0.9 Gb, *Limnodynastes ornatus* = ornate burrowing frog) to among the largest known in animals (118 Gb, *Necturus lewisi* = gulf coast waterdog; Gregory, 2012). The estimated genome sizes and chromosome numbers of commonly used amphibian species in toxicology are presented in **Table 2**. Thus, coupling toxicological demands with genetics result in logistical and bioinformatic challenges. These have hampered building consensus and concerted effort to further genomics tools.

Despite this, current efforts are focused upon addressing the dearth in (1) application of available toxicogenomics resources to amphibians, and (2) genome sequence information representing amphibian species beyond *Xenopus*. Access to genome

Table 2 | Estimated genome sizes[a] of representative amphibians.

Species (common name)	Genome size (Gb)	Chromosome number
ANURA		
Bombina orientalis (oriental fire-bellied toad)	8.0	24
Bufo americanus (American toad)	5.1	22
Bufo bufo (common toad)	6.6	22
Bufo marinus (cane toad)	4.8	22
Hyla arborea (European tree frog)	4.7	24
Hyla versicolor (gray tree frog)	9.6	48
Pelobates fuscus (European spadefoot toad)	4.4	26
Pseudacris regilla (Pacific tree frog)	3.7	24
Rana aurora (red-legged frog)	9.0	26
Rana catesbeiana (North American bullfrog)	7.4	26
Rana clamitans (green frog)	6.7	26
Rana esculenta (edible frog)	6.8	26
Rana japonica (Japanese reddish frog)	5.7	26
Rana pipiens (northern leopard frog)	6.7	26
Rana rugosa (wrinkled frog)	8.0	26
Rana sylvatica (wood frog)	5.8	26
Rana temporaria (common European frog)	4.2	26
Spea hammondii (Western spadefoot toad)	1.6	26
Xenopus laevis (South African clawed frog)	3.2	36
Xenopus tropicalis (Western clawed frog)	1.7	20
CAUDATA		
Ambystoma maculatum (spotted salamander)	32.3	28
Ambystoma mexicanum (Mexican axolotl)	34.0	28
Ambystoma tigrinum (tiger salamander)	31.0	28
Andrias japonicus (Japanese giant salamander)	45.5	60
Dicamptodon ensatus (Pacific giant salamander)	55.6	28
Necturus maculosus (mudpuppy)	84.1	38
Notophthalmus viridescens (red spotted newt)	36.9	22
Pleurodeles waltl (Spanish ribbed newt)	20.0	24
Triturus vulgaris (common newt)	24.9	24
GYMNOPHIONA		
Geotrypetes seraphini (Gaboon caecilian)	4.6	38
Gymnopis multiplicata (Purple caecilian)	3.6	24–26
Siphonops annulatus (caecilian)	13.6	?

Adapted from Gregory (2012).

[a] *Genome sizes presented are the average of C-values from the Animal Genome Size database for a given species. C-values represent the haploid DNA amount in a gametic nucleus. The term is used interchangeably with genome size for diploids. However, when an organism is polyploid, the C-value may represent multiple genomes within the nucleus and may not represent the true haploid DNA amount.*

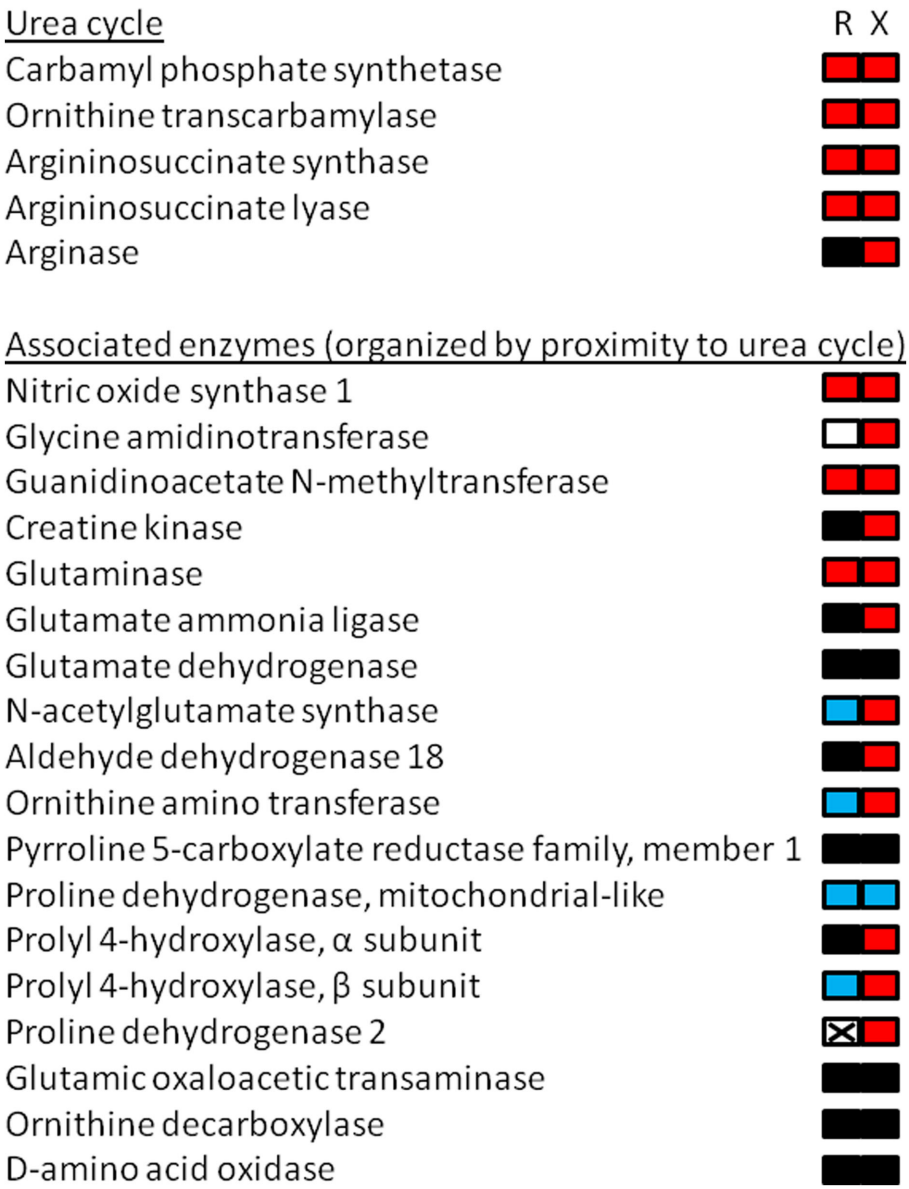

FIGURE 1 | Cartoon depiction of RNA-seq results from the liver of premetamorphic *Xenopus laevis* and *Rana catesbeiana* tadpoles focusing upon arginine and proline metabolism including the urea cycle. Tadpoles were exposed to 10 nM 3,5,3′-triiodothyronine (a thyroid hormone) or NaOH vehicle control for 48 h. The animals were treated and maintained in accordance with the guidelines of the Canadian Council on Animal Care. The liver transcriptomes were subjected to RNA-seq using 75 base HiSeq of paired end tagged (PET) libraries. The derived sequence information was assembled using the *X. tropicalis* genome as a scaffold and the contig identities were determined by a Blastx search against the *X. tropicalis* genome. The number of read counts (∼400 million) was normalized between samples and the relative count frequencies of the indicated pathway components were compared based upon the *X. tropicalis* arginine and proline metabolism KEGG pathway (xtr00330; www.genome.jp/kegg). The results are depicted as a bipartite rectangle beside the name of the enzymes corresponding with measured transcripts in the RNA-seq experiment that were identified in the KEGG pathway. The left side represents the relative change in transcript abundance levels of *Rana* (R) and the right side mRNA levels of *Xenopus* (X) where red is increased, black is no change, and blue is decreased relative to control animals. Non-detected transcript is depicted by a crossed-out white box. Use of the *X. tropicalis* genome as an assembly scaffold had limited utility since *X. laevis* and *R. catesbeiana* sequences aligned imperfectly to the *X. tropicalis* genome with *R. catesbeiana*, not surprisingly, having the least benefit of alignment. Nevertheless, some transcript identities linked to count frequencies were positively confirmed and the data obtained for the urea cycle enzymes, for example, matched well with previous observations (Helbing et al., 1992; Xu et al., 1993; Iwase et al., 1995). This validates the method for transcripts that are identifiable and quantifiable in this way.

and transcriptome sequence information is critical for the key toxicogenomics approaches today such as microarrays, QPCR, and proteomics techniques. The increasingly recognized role of epigenetic factors in toxicology necessitates the means for

analyzing genomes (Bilesimo et al., 2011). *De novo* high throughput sequencing of transcriptomes (RNA-seq) provides an unprecedented opportunity to obtain sequence and expression information of literally thousands of gene transcripts within a tissue (Martin and Wang, 2011). Access to resources such as Xenbase (www.xenbase.org) and deposition of amphibian expressed sequence tags (ESTs) and individually cloned sequences on publicly accessible databases have made it possible to garner limited information from RNA-seq experiments (**Figure 1**). However, accurate assembly and bioinformatics evaluation of RNA-seq data requires a genome sequence for the species of interest.

We will see more use of *X. tropicalis* for toxicogenomics; but we must be very careful not to let the genomics drive the toxicology and put resources and effort into developing appropriate tools for a wider range of toxicologically relevant species. Of the greatest urgency is obtaining genome sequence information from the Ranids, since they represent the largest group of amphibians worldwide, and the Caudata due to their unique genome structure. Now more than ever, we need the scaffolds and platforms for more amphibian species to move toxicogenomics forward.

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