

Sediment TCDD-EQs and EROD and MROD Activities in Ranid Frogs from Agricultural and Nonagricultural Sites in Michigan (USA)

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Received: 18 July 2005 / Accepted: 26 December 2005

Abstract. *In vitro* studies have demonstrated atrazine-mediated induction of 7-ethoxyresorufin *O*-deethylase (EROD) activity. EROD is an enzyme active in the metabolism of many compounds, including many xenobiotics. These studies have suggested that atrazine may affect reproductive function by altering steroid metabolism. The goal of this study was to determine whether relationships could be detected between measured atrazine concentrations in surface waters and the liver-somatic index (LSI) and EROD and 7-methoxyresorufin *O*-deethylase (MROD) activities in the livers of ranid frogs. In addition, sediment dioxin toxic equivalents (TCDD-EQs) were determined using the H4IIE-luc cell bioassay. Adult and juvenile green frogs (*Rana clamitans*), bullfrogs (*R. catesbeiana*), and Northern leopard frogs (*R. pipiens*) were collected from areas with extensive corn cultivation and areas where there was little agricultural activity in south central Michigan in the summer of 2003. Atrazine concentrations at nonagricultural sites ranged from less than the limit of quantification (0.17 µg atrazine/L) to 0.23 µg atrazine/L and did not exceed 1.2 µg atrazine/L at agricultural sites. Sediment TCDD-EQs were measurable only at one agricultural site. Of the measured parameters, only LSI values in adult male frogs differed significantly between agricultural and nonagricultural sites, with greater values observed at agricultural sites. In green frogs, EROD and MROD activities were measurable in both adult and juvenile frogs and were similar among sites. Median EROD activities ranged from 13 to 21 pmol/min/mg protein in adult male green frogs and from 5 to 13 pmol/min/mg protein in adult female green frogs. Juvenile frogs had greater EROD and

MROD activities than adult frogs. Bullfrogs and leopard frogs had greater activities than did green frogs. Atrazine concentrations were significantly and negatively correlated with MROD activity in adult male green frogs (Spearman $R = -0.800$). LSI and EROD and MROD activities of adult female or juvenile green frogs were not significantly correlated with atrazine concentrations. These results suggest that atrazine does not appear to have a consistent association with EROD or MROD activities in wild-caught green frogs.

The triazine herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is an herbicide globally used on crops such as corn, sorghum, and sugar cane (United States Environmental Protection Agency 2003). In the Midwestern United States, atrazine is used in corn production. It is typically applied in the spring, when many amphibians are breeding in aquatic habitats that may receive runoff from agricultural fields. Atrazine has recently become the subject of great interest because research has suggested a link between atrazine exposure and disruption of normal sexual development in frogs (Hayes *et al.* 2002; Hayes *et al.* 2003). The mechanism by which atrazine has been proposed to influence reproductive development in frogs is by way of atrazine-mediated upregulation of aromatase (*CYP19*), the cytochrome p450 enzyme that converts testosterone to estradiol (Hayes *et al.* 2002; Hayes 2005). Evidence for this mechanism was provided by two studies using the mammalian cell line H295R (Sanderson *et al.* 2000, 2001). However, in a separate study, atrazine did not affect the expression of aromatase in a rat cell line, R2C (Heneweer *et al.* 2004). To date, there are no reports

of atrazine altering either aromatase activity in juvenile or adult African clawed frogs (*Xenopus laevis*) (Coady *et al.* 2005; Hecker *et al.* 2005b, c) or expression of *CYP19* mRNA in adult *X. laevis* (Hecker *et al.* 2005b).

Other studies have indicated that atrazine may modulate the endocrine system indirectly through other enzyme systems. A recent *in vitro* study found that concentrations as small as 5 nM atrazine (1.08 µg/L) significantly inhibited phosphodiesterase activity and resulted in an increase of the second messenger cAMP, which the investigators hypothesized had the potential to induce aromatase activity (Roberge *et al.* 2004). A study with mammalian cells found that atrazine induced 7-ethoxyresorufin *O*-deethylase (EROD) activity, an effect that the investigators suggested could result in increased estrogen metabolism (Oh *et al.* 2003). A study of triazine metabolism in rat liver microsomes found significant correlations between EROD activity and the formation of atrazine metabolites, indicating that EROD played a role in atrazine metabolism, and therefore, possibly in atrazine-mediated toxic effects (Hanioka *et al.* 1999).

EROD activity is directly associated with the induction of hepatic activity of the cytochrome P4501A1 enzyme, a mixed-function oxygenase (MFO) enzyme that is part of the cytochrome P450 enzyme superfamily and whose main function is the metabolism of xenobiotics. In mammals, the P4501A subfamily is represented by two members, P4501A1 and P4501A2, which share approximately 72% amino-acid sequence identity but display different substrate specificities and inhibitor susceptibilities (Kawajiri and Hayashi 1996). P4501A1 mainly metabolizes polycyclic aromatic hydrocarbons (PAHs) and structurally similar chemicals, whereas P4501A2 preferentially oxidizes heterocyclic and aromatic amines. P4501A1 is much more effective at metabolizing 7-ethoxyresorufin (ER), whereas P4501A2 is more effective at metabolizing 7-methoxyresorufin (MR), but the fact that both enzymes metabolize some substrates makes discrimination of their activities difficult. However, metabolism of both substrates provides valuable information regarding P4501A activity in an organism relative to its exposure to xenobiotics. Two members of the CYP1A family, CYP1A6 and CYP1A7, have been identified in *X. laevis*, each of which shares approximately 60% sequence identity with mammalian CYP1A1 and CYP1A2, respectively (Fujita *et al.* 1999), and both of which are induced by model chemicals such as 3-methylcholanthrene (Colombo *et al.* 2003).

Induction of EROD is commonly used as a functional measure of exposure of vertebrates to halogenated aromatic hydrocarbons such as dioxins, polychlorinated biphenyls (PCBs), and PAHs (Andersson *et al.* 2000; Kennedy *et al.* 1996; Smeets *et al.* 2002). MFO activities in amphibians, although not yet as well studied as in other vertebrates, have been measured in both laboratory and field studies. Several of these studies indicated that EROD activity in some species of amphibians can be induced to levels of activity similar to those that have been observed in rats and other mammals, but these responses can be variable and are associated with species, sex, and stage of development (Ertl and Winston 1998; Gauthier *et al.* 2004; Longo *et al.* 2004). For example, *X. laevis* has lesser MFO activities than those measured in ranid frogs such as the bullfrog (*Rana catesbeiana*) and leopard frog (*R. pipiens*) (Ertl and Winston 1998).

In addition to EROD activity, methoxyresorufin *O*-methylase (MROD) activity has been used as another hepatic biomarker of exposure in frogs. Although not as highly inducible as EROD, MROD activity has been found to be a more sensitive end point than other monooxygenase enzymes, such as benzyloxy-ROD and pentoxy-ROD, in amphibians (Huang *et al.* 1998). Consequently, the aims of this study were to measure EROD and MROD activities as indicators of CYP1A6 and CYP1A7 expression in ranid frogs collected from agricultural and nonagricultural areas and to investigate site differences and correlations with measured concentrations of atrazine in surface waters. In addition, because dioxins and dioxin-like compounds are known inducers of EROD activity, sediment AhR activity was determined using a cell bioassay to calculate sediment 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQs) at the study sites.

Materials and Methods

Site Selection and Frog Sampling

Site selection, frog sampling procedures, and atrazine concentrations have been described in detail elsewhere (Murphy *et al.* 2006). Briefly, agricultural (corn growing) and nonagricultural sites were sampled twice during the summer of 2003. Sites were located in two regions in south-central Michigan: Kalamazoo and the Greater Lansing Area (Fig. 1). Nonagricultural sites were located in both regions. Study sites were selected based on atrazine concentrations and the presence of relatively large populations of ranid frogs. Detailed site descriptions can be found in Murphy *et al.* (2006) and Murphy (2005).

Atrazine concentrations were measured in water samples collected in 1-liter water-and-methanol-rinsed amber glass I-Chem bottles (Fisher Scientific, Hampton, NH) approximately monthly from May to September. Samples were tested for atrazine concentrations using Envirogard triazine enzyme-linked immunosorbent assay (ELISA) kits (Strategic Diagnostics, Newark, DE). All samples underwent solid-phase extraction (SPE) using 5 ml SPE cartridges (AnSys Technologies, Palo Alto, CA) to remove humic and fulvic acids before use in the triazine ELISA. The extraction and ELISA procedures were validated using blanks and atrazine-spiked samples. The method detection limit (MDL) of the ELISA was 0.05 µg atrazine/L, whereas the limit of quantification (LOQ), defined as 2 SD greater than the MDL, was 0.17 µg atrazine/L.

The main species of interest in this study was the green frog (*R. clamitans*), which is the most common pond frog in Michigan (Harding 1997). Green frogs are territorial breeders associated with the aquatic environment throughout their lives and are highly faithful to their pond or wetland habitats (Martof 1953; Harding 1997). Green frogs may breed more than once during the breeding season; tadpoles that hatch late in the breeding season overwinter in pond sediments, whereas those that hatch earlier in the summer metamorphose into adults (Harding 1997). Whether a juvenile frog was from the previous or current year was readily apparent based on body size, making it possible to collect juveniles from the current year with a high degree of certainty that they were from egg masses laid earlier that summer. In addition, study sites were visited at least four times during the summer, which allowed the breeding status of the frog populations at each site to be monitored. However, other ranid species, including bullfrogs (*R. catesbeiana*) and leopard frogs (*R. pipiens*), were collected as well to investigate interspecies differences in the measured biomarkers. Adult frogs were sampled in May and juvenile frogs were collected in July. Site Ag2 had the largest frog population among the agricultural sites and was resampled for adult frogs in September to

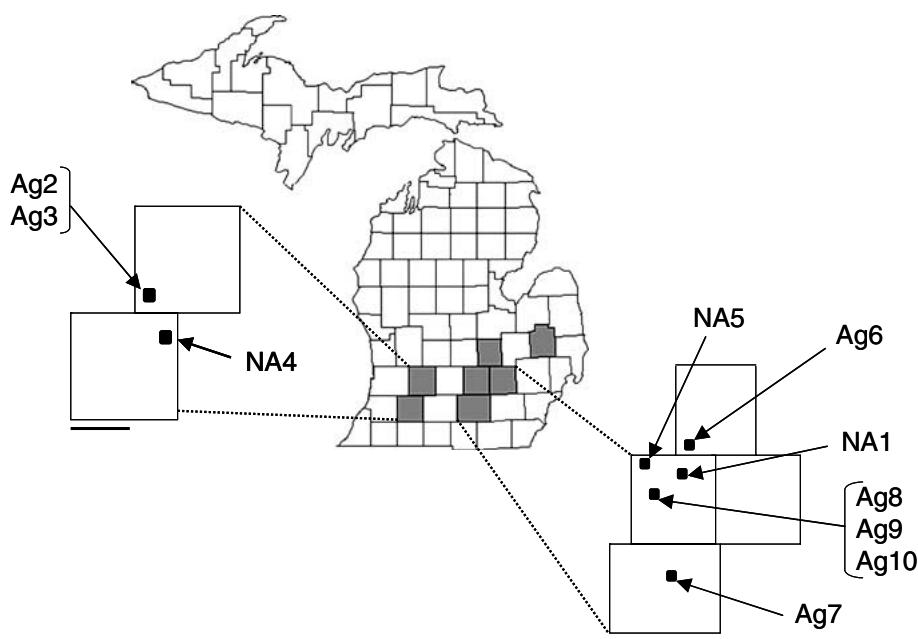


Fig. 1. Study site locations for frog collection. Sites were located in two regions, near Kalamazoo and in the greater Lansing area of south-central Michigan, in both agricultural (“Ag”) and nonagricultural areas (“NA”). Bar = 20 km

determine how the measured parameters differed at the end of the breeding season.

Frogs were collected at night using hand nets and buckets. The target sample size for each sampling event was between 40 and 50 frogs/site/age class, and the minimum number collected was 22 frogs. Frogs were anesthetized in tricaine methanesulfonate (MS-222, 500 mg/L) and sacrificed by cervical dislocation. All procedures involving animals were approved by and conducted in accordance with policies set forth by the All-University Committee on Animal Use and Care at Michigan State University under an approved animal use permit. Livers were removed, weighed, and flash-frozen in liquid nitrogen. The LSI was calculated as follows:

$$\text{LSI} = (\text{liverweight/bodyweight}) \cdot 100$$

Measurement of MFO Activities

Liver microsomes were prepared and EROD and MROD activities were measured using methods described by Kennedy and Jones (1994) with modifications. Briefly, 0.5 g liver tissue was homogenized in Tris buffer (0.05 M Tris and 1.15% KCl, pH 7.5) centrifuged to obtain the microsomal fraction, resuspended in microsomal stabilization buffer (20% glycerol, 0.1 M KH_2PO_4 , 1 mM ethylene diamine tetraacetic acid, and 1 mM dithiothreitol at pH 7.25) and stored at -80°C . Livers collected from juvenile frogs weighed < 0.5 g with few exceptions, and therefore the entire liver was used in microsome preparation. EROD and MROD assays were optimized on 96-well plates (Corning Life Sciences, Corning, NY) for all species and age classes collected. The working range for both the EROD and MROD assays was 0 to 120 pmol resorufin/well, and all assays were conducted with 12 μg microsome preparation/well in 0.05 M HEPES buffer (pH 7.8). EROD assays were conducted with 0.3 mM nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma, St. Louis, MO) and 15 μM ethoxyresorufin (ER)/well. MROD assays were conducted with 0.45 mM NADPH and 7.5 μM methoxyresorufin (MR)/well. ER and MR were obtained from Molecular Probes (Eugene, OR). All assay plates were preincubated for 10 minutes at 30°C , which was determined to be the optimum incubation temperature for EROD and MROD enzyme activities in all species, before

NADPH addition. EROD and MROD assays were incubated at 30°C for 10 and 15 minutes, respectively, after which the reaction was stopped using acetonitrile. EROD and MROD activities were expressed as pmol substrate converted per min per mg protein. Protein concentrations were measured using the Bradford assay with bovine serum albumin (Sigma) as the protein standard (Bradford 1976). Nondetectable activities were set to values equaling one half of the lowest detectable enzyme activity. These values were 1.07 and 0.65 pmol/min/mg protein for EROD and MROD in adults, respectively, and 1.46 and 0.84 pmol/min/mg protein in juveniles, respectively.

Sediment Extraction

Sediments were extracted using methods described elsewhere (Kannan *et al.* 2000). Briefly, 20 g sediment was dried by mixing with sodium sulfate and Soxhlet extracted using dichloromethane (DCM) and hexane (3:1, 400 ml). Extracts were treated with activated copper coils to remove sulfur, rotavapped to near-dryness, transferred to calibrated test tubes, and evaporated to 11 ml under nitrogen. One ml extract was removed for use in cell bioassays, and the remaining extract was passed through glass columns containing 10 g Florisil for fractionation. The first fraction was eluted with 100 ml hexane and was expected to contain nonpolar compounds such as PCBs. The second fraction was eluted with 100ml 20% DCM in hexane and was expected to contain PAHs, polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, and some organochlorine pesticides. The third fraction was eluted with 50% DCM in methanol and was expected to contain polar compounds such as alkylphenols and some pesticides, including triazine compounds such as atrazine. All fractions were evaporated to 1 ml in either hexane or methanol for use in cell bioassays.

Cell Culture and AhR Bioassay

H4IIE-luc cells are rat hepatoma cells that express the AhR receptor and have been stably transfected with a luciferase reporter gene under the control of dioxin-responsive elements (Sanderson *et al.* 1996).

Table 1. Atrazine concentrations measured in water samples collected at study sites in 2003 using a triazine ELISA^a

Site	Atrazine Concentration ($\mu\text{g/L}$)				
	May	June	July	August	September
NA1	< 0.17	< 0.17	< 0.17	< 0.17	< 0.17
NA4	< 0.17	< 0.17	< 0.17	0.23	< 0.17
NA5	– ^a	< 0.17	< 0.17	< 0.17	< 0.17
Ag2	0.20	< 0.17	< 0.17	< 0.17	< 0.17
Ag3 ^b	0.19	0.39	0.18	–	–
Ag6	< 0.17	0.19	0.27	< 0.17	–
Ag7	0.18	–	< 0.17	< 0.17	< 0.17
Ag8	0.21	0.20	0.29	0.18	1.0
Ag9 ^c	–	0.70	0.73	0.63	0.45
Ag9 ^c	–	1.12	1.17	0.98	0.70
Ag10	–	0.21	0.33	< 0.17	< 0.17

^a – = No water sample collected at time point.

^b Site Ag3 dried up in late summer.

^c This site was composed of two ponds with different atrazine concentration profiles.

Cells were cultured at 37°C and 5% carbon dioxide. Cell bioassays were conducted as described elsewhere (Hilscherova *et al.* 2000). Briefly, 250 μl cell solution/well was seeded into the inner 60 wells of 96-well culture plates (Perkin Elmer, Boston, MA). H4IIIE-luc cells were seeded at a density of approximately 80,000 cells/ml. Cells were incubated for 24 hours and then exposed to extraction fractions at 1% v/v in triplicate. Solvent controls and blanks were included on all plates. Cells were incubated for 72 hours and then used in the luciferase assay with 50 μl Luc-Lite reagent (Perkin Elmer)/well. Sample responses were solvent corrected and compared with dioxin standards ranging from 0.48 to 1500 pM and expressed as pg TCDD-EQ (Coady *et al.* 2001).

Statistical Methods

Data were tested for normality using the Kolmogorov-Smirnov test with Lilliefors transformation and probability plots. LSI, EROD, and MROD activities were normalized using ln (natural log) transformation, and significant differences among sites were determined using analysis of variance (ANOVA). A two-sample Student *t*-test and Bonferroni adjustment were used to determine differences between agricultural and nonagricultural sites. If data could not be ln-normalized, significant differences between agricultural and nonagricultural sites were determined using nonparametric Kruskal-Wallis and Mann-Whitney *U*-tests. The rank-order Spearman test was used to test for correlations between atrazine concentrations and median LSI and median EROD and MROD activities. A one-tailed Dunnett's test was used with H4IIIE-luc data to determine the first concentration that differed from zero if sample dose-response curves did not meet bioassay assumptions approximately regarding parallelism and efficacy (Villeneuve *et al.* 2000). All analyses were conducted using Systat 11 (SSI, Richmond, CA). Significance level was set at $p < 0.05$ for all statistical tests.

Results

Because of the larger number of green frogs, statistical analyses were conducted primarily on this species. However, comparisons with bullfrogs and leopard frog were made whenever possible to attempt to determine interspecies differences in the measured biomarkers.

Atrazine Concentrations in Pond Water

Atrazine concentrations measured at sites designated as agricultural ranged from less than the LOQ (0.17 μg atrazine/L) to 1.0 μg atrazine/L (Table 1). Atrazine concentrations at non-agricultural sites ranged from 0.17 to 0.23 μg atrazine/L. Mean concentrations of atrazine were significantly greater at agricultural sites compared with nonagricultural sites ($p = 0.010$, Mann-Whitney *U*).

AhR Activity

Sediment TCDD-EQs were < 1 pg/g dry weight (dw) at all sites with the exception of site Ag2. Dioxin-like activity, when measurable, was observed in the second and third fractions of the extracted sediments. Only fractions II and III from site Ag2 produced concentration-response curves from which EC₅₀ values could be derived. These values were determined to be 4.64 pg TCDD-EQ/g and 4.20 pg TCDD-EQ/g dry wt, respectively. Dioxin-like activity was not detectable at any of the other study sites.

LSI

LSI values were comparable between adult and juvenile frogs, but overall LSI values were greater in adult female frogs than in male adult or in male or female juvenile frogs (Figs. 2 through 5). The greatest median LSI was measured in adult female green frogs at site Ag6 (LSI = 3.88). LSI differed significantly among sites in both male and female adult green frogs and was significantly greater at agricultural sites in adult male green frogs (Table 2 and Figs. 2 and 3). In juvenile green frogs, LSI was significantly different among sites but did not differ between agricultural and nonagricultural sites (Table 2 and Figs. 4 and 5). Power analysis indicated that a 1.25-fold change in LSI was detectable in adult female green frogs at a power $(1-\beta) = 0.23$ and in juvenile green frogs at a power < 0.13.

LSI values in adult bullfrogs were comparable with those measured in adult green frogs. However, LSI values were

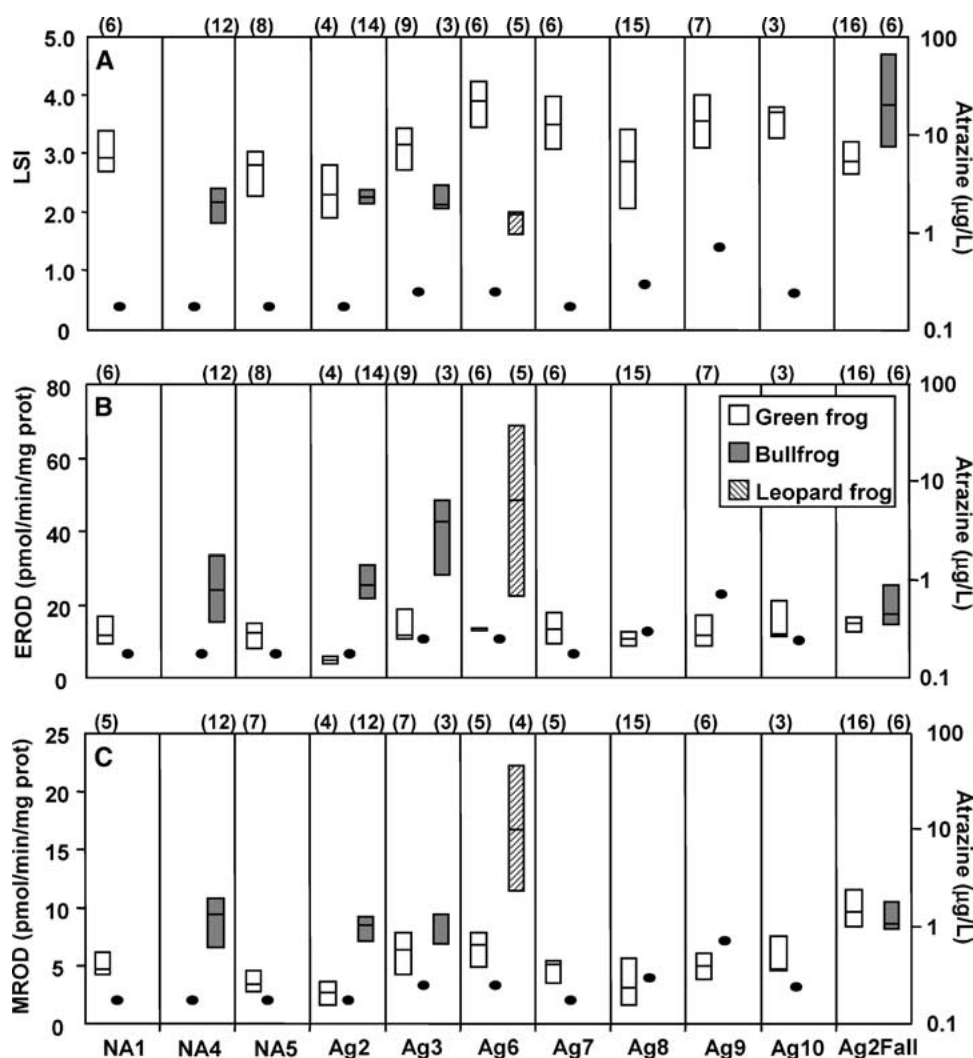


Fig. 2. (A) LSI, (B) EROD, and (C) MROD activities measured in adult female frogs. The horizontal line on each bar represents the median, and bar length represents the middle 50% of the data. The numbers in parentheses above the graphs represent sample size for each species. Points represent the third quartile of the atrazine concentrations measured in water from the sites

Table 2. *p* values for intersite and site region comparisons in adult and juvenile green frogs

	Sex	Age Class	LSI	EROD	MROD
Intersite differences ^a	Male	Adults	< 0.001	0.418	0.016
		Juveniles	< 0.001	0.082	< 0.001
	Female	Adults	0.029	0.044	0.056
		Juveniles	< 0.001	0.057	0.213
Nonagricultural vs. agricultural ^b	Male	Adults	0.001	0.144	0.871
		Juveniles	0.079	0.432	< 0.001
	Female	Adults	0.343	0.978	0.738
		Juveniles	0.162	0.433	0.085

^a Intersite differences were determined using ANOVA in adults, and Kruskal-Wallis tests in juveniles.

^b Differences between agricultural and nonagricultural sites were determined using student *t*-tests in adults, and Mann-Whitney *U*-tests in juveniles.

greater in adult female bullfrogs than in adult female green frogs at the site that was resampled in the fall (Fig. 2). The LSI values of both juvenile and adult female leopard frogs were less than the LSI values of juvenile green frogs or adult female bullfrogs and green frogs. The LSI of adult male leopard frogs was comparable with the other two species.

EROD and MROD Activities

EROD activities were comparable between adult male and female green frogs, whereas EROD activity was greater in juvenile compared with adult frogs. The greatest median EROD activity in both adult and juvenile green frogs was

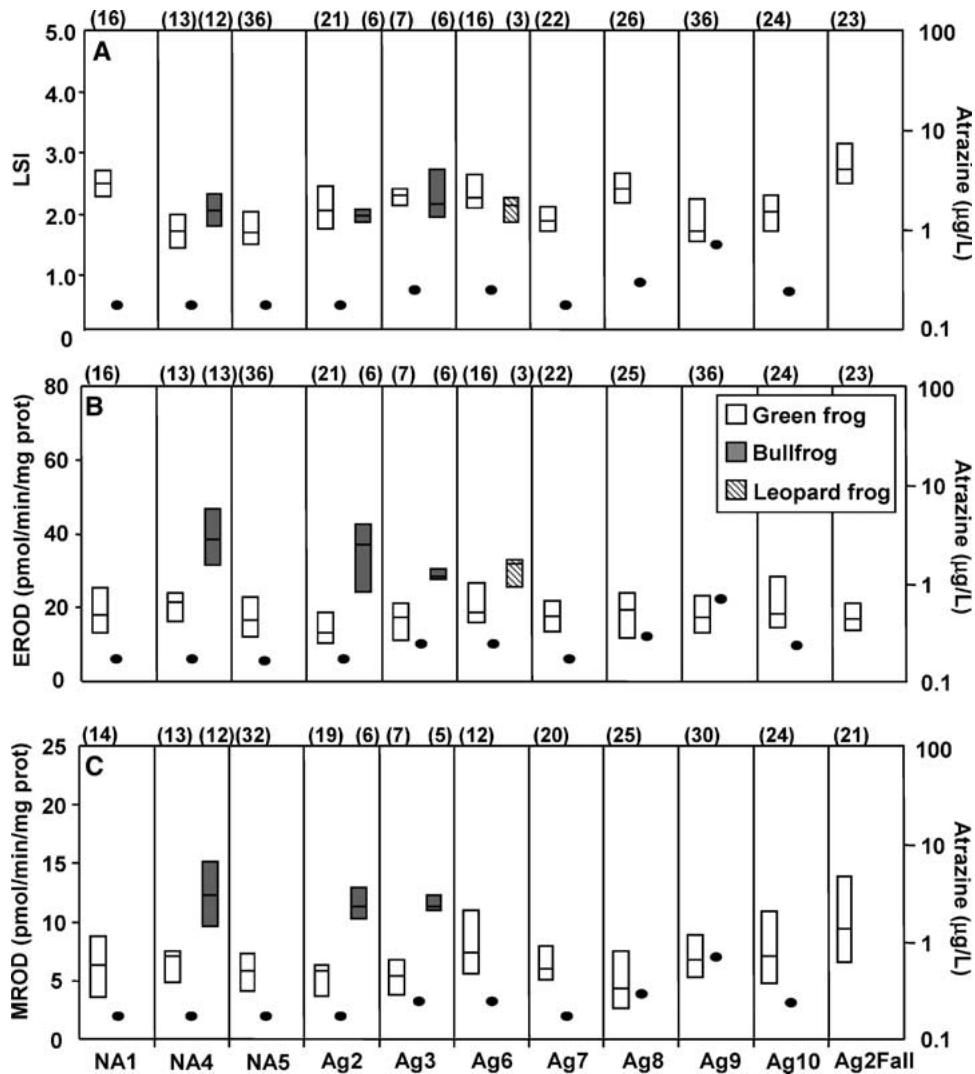


Fig. 3. (A) LSI, (B) EROD, and (C) MROD activities measured in adult male frogs. The horizontal line on each bar represents the median, and bar length represents the middle 50% of the data. The numbers in parentheses above the graphs represent sample size for each species. Points represent the third quartile of the atrazine concentrations measured in water from the sites

measured in male frogs at site NA4 (EROD activity = 21.4 and 29.8 pmol/min/mg protein, respectively; Figs. 3 and 4). EROD activities were not significantly different among sites or between agricultural and nonagricultural regions in both male and female adult frogs (Table 2 and Figs. 2 and 3). Similarly, EROD activities in juvenile male and female green frogs also did not differ significantly among sites or between agricultural and nonagricultural regions ($p = 0.433$ Mann-Whitney U ; Figs. 4 and 5).

In general, MROD activities were twofold to threefold less than EROD activities and were greater in juvenile green frogs compared with adult frogs (Figs. 2 through 5). However, it is important to note that MROD activities were less than the assay detection limit in 25% of juvenile female frogs and in 30% of juvenile male frogs. The greatest median activity in adults was measured in male green frogs at site Ag6 (MROD activity = 7.4 pmol/min/mg protein). MROD activities were significantly different among sites in adult male green frogs, but activities were not significantly different among sites in adult female frogs or between agricultural and nonagricultural regions in both male and female frogs (Table 2 and Figs. 2 and 3). In juvenile female green frogs, MROD activity was not significantly different among sites or between agricultural and

nonagricultural regions (Table 2 and Fig. 4). MROD activity in juvenile male green frogs was significantly different among sites and was significantly greater at agricultural sites region (Table 2 and Fig. 5).

The EROD and MROD activities measured in bullfrogs and leopard frogs were greater than those measured in adult and juvenile green frogs (Figs. 3 and 4). Furthermore, EROD and MROD activities in juvenile male leopard frogs were as much as twofold and fourfold greater, respectively, than activities in juvenile male green frogs.

A twofold difference in EROD or MROD activity was detectable in adult male green frogs at a statistical power ($1-\beta$) of 0.74 and 0.68, respectively. The statistical power to detect this difference in adult female green frogs was approximately 0.30 for both EROD and MROD activities and in both male and female juvenile green frogs was approximately 0.05.

Correlations Among Parameters

Median LSI was significantly and positively correlated with both median EROD (Pearson $R = 0.632$, $p < 0.050$) and median MROD (Pearson $R = 0.699$, $p = 0.025$) activities in

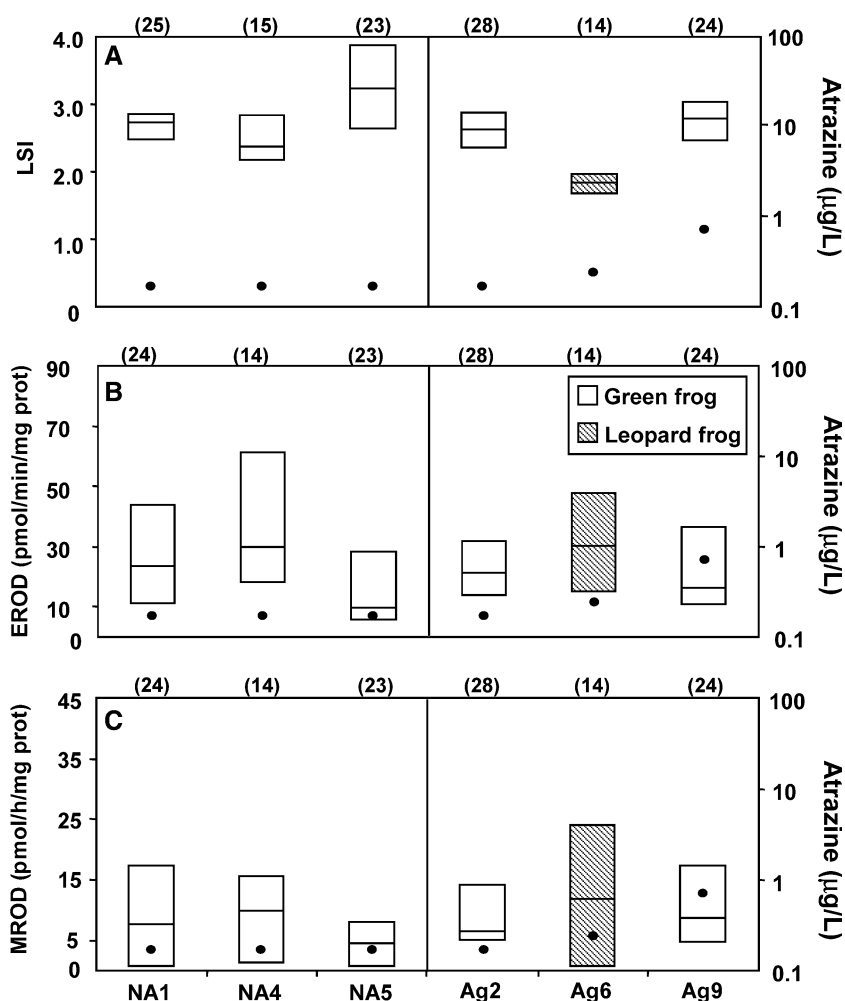


Fig. 4. (A) LSI, (B) EROD, and (C) MROD activities measured in juvenile female frogs. The horizontal line on each bar represents the median, and bar length represents the middle 50% of the data. The numbers in parentheses above the graphs represent sample size for each species. Points represent the third quartile of the atrazine concentrations measured in water from the sites. Juvenile frogs were not encountered at all sites where adults were collected.

adult female green frogs but was not correlated with either median EROD or MROD activity in adult male green frogs. In juvenile female green frogs, median EROD activity was significantly and negatively correlated with median LSI (Pearson $R = -0.900$, $p = 0.002$). Median MROD activity was also negatively related to median LSI, but the correlation was not significant (Pearson $R = -0.664$, $p = 0.073$). Median LSI was negatively and significantly correlated with EROD activity in juvenile male green frogs (Pearson $R = -0.788$, $p = 0.020$) but was not correlated with median MROD activity.

Exposure Correlations

Correlations between atrazine concentrations measured in water from the study sites and the medians of the measured parameters were determined based on concentrations measured both 4 weeks before sampling (T-28) and at the time of sampling (T-0). Median LSI and EROD and MROD activities were not correlated with T-28 or T-0 in adult female green frogs. Median MROD activity was negatively correlated with T-28 in adult male green frogs (Spearman $R = -0.800$), but neither median EROD nor LSI was correlated with atrazine concentration. No significant correlations were observed

between atrazine concentration and LSI or EROD or MROD activities in either juvenile male or female green frogs.

Of the sites evaluated in this study, only site Ag2 sediments contained measurable TCDD-EQ concentrations. LSI and EROD and MROD activities were not significantly different at this site compared with the other study sites for either juvenile male or female green frogs. In adult female green frogs, EROD activity was significantly less at site Ag2 compared with the other sites ($p < 0.017$, ANOVA), although sample size at this site was small ($n = 4$). No relations were observed between TCDD-EQs and LSI, EROD, or MROD activities in adult male green frogs.

Discussion

EROD and MROD activities were measurable in all three frog species and in both age classes. EROD activities were significantly different across sites in adult female green frogs, whereas MROD activities of adult and juvenile male green frogs were significantly different among sites. LSI showed the greatest among-site variability of all the parameters measured and was found to differ significantly among sites for green frogs of both sexes and age classes. The positive correlations

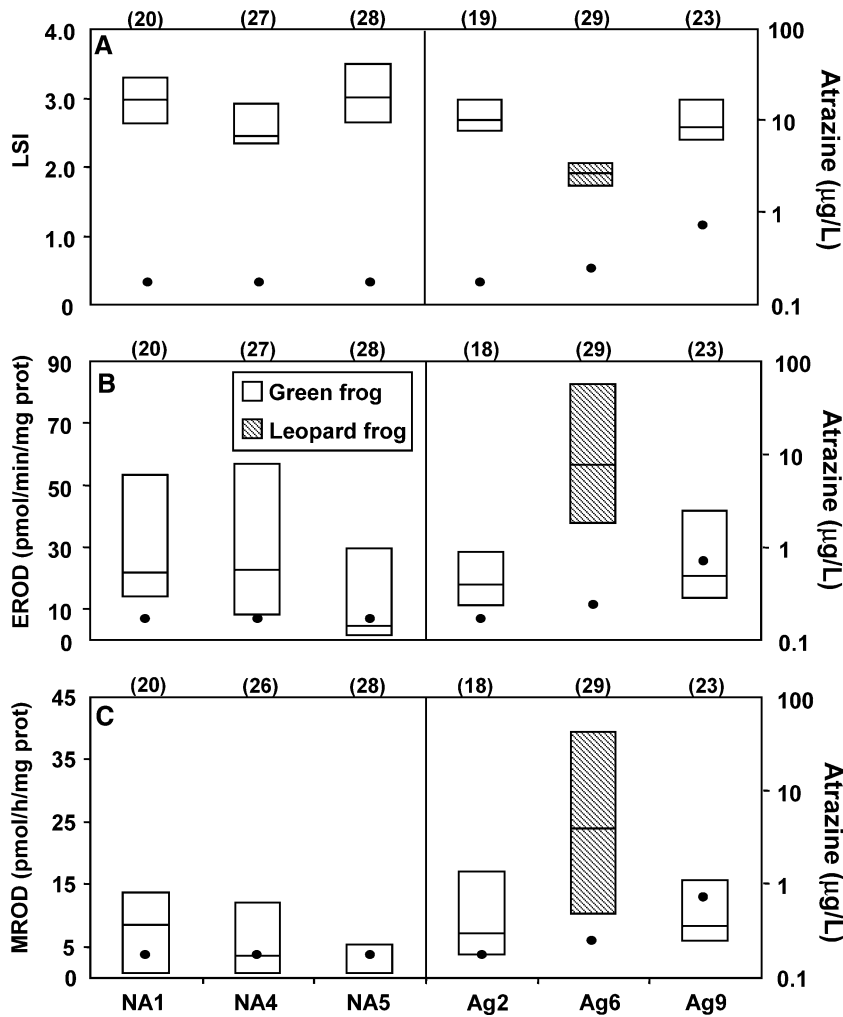


Fig. 5. (A) LSI, (B) EROD, and (C) MROD activities measured in juvenile male frogs. The horizontal line on each bar represents the median, and bar length represents the middle 50% of the data. The numbers in parentheses above the graphs represent sample size for each species. Points represent the third quartile of the atrazine concentrations measured in water from the sites. Juvenile frogs were not encountered at all sites where adults were collected.

observed between LSI and MROD and EROD activities in adult female green frogs are consistent with previously reported observations that induction of MFO enzymes resulted in greater liver weight. The reason for the lack of correlation between these parameters in male and the negative correlations between EROD and LSI in juvenile frogs remains unclear. However, increase of relative liver size is a general indicator of increased metabolic activity or other biosynthetic processes such as yolk protein synthesis (Rinchar and Kestemont 2003), and some of the differences in LSI may be caused by such metabolic activities that were not captured in this study. The greater LSI in female compared with male frogs was likely caused by vitellogenesis occurring in the liver in female green frogs during early summer before the spawning season, a process that has been reported to be positively correlated to liver size in fish (Hecker *et al.* 2002).

EROD and MROD activities measured in frogs collected in September at site Ag2 were similar to the activities measured in May and June, which was unexpected. Studies of fish have found an increase in EROD activity in female frogs after spawning, whereas EROD activity decreased in male frogs after spawning (Lange *et al.* 1999). Activities in frogs were therefore expected to increase at the end of the summer in female frogs, because the tissue that was built up for

reproduction was being metabolized, and frogs were preparing to hibernate (Rinchar and Kestemont 2003). It is possible that frogs sampled in September were near the end of their breeding season but had not yet begun to metabolize reproductive tissue. It is also possible that in frogs, EROD and MROD do not play major roles in this type of metabolism, in which case no increase in activity would occur.

The ranges in EROD and MROD activities measured in adult frogs in this study are similar to those observed in other studies, but there are also ecological and interspecies differences in these enzyme activities. A field study in orchard wetlands found mean EROD activities ranging from 10 to 35 pmol/min/mg protein in adult male green frogs and from 40 to 60 pmol/min/mg protein in adult male leopard frogs (Harris *et al.* 1998). In contrast, a field study on EROD activity in green frog tadpoles and metamorphs measured approximately 5- and 10-fold lesser EROD activities, respectively, than those measured in the current study (Jung *et al.* 2004). An interspecies comparison of antioxidant and MFO enzymes found a mean EROD activity of 34 nmol/min/mg protein in bullfrogs (Rocha-e-Silva *et al.* 2004), an activity that is 1000-fold greater than those measured in this study. The same study found a mean EROD activity of 238 nmol/min/mg protein in the cane toad *Bufo marinus*. However, this activity was

measured in field-collected toads in Brazil, so it is possible that this greater EROD activity was caused by a combination of species differences as well as differences in metabolic rate in temperate and tropical organisms. The disparities in EROD and MROD activities measured in populations and individuals of the same species may indicate that site-specific environmental factors influence metabolic activity.

Although studies have shown that EROD induction may be a useful biomarker of effect in reptiles, including snakes (Hecker *et al.* 2006) and alligators (Gunderson *et al.* 2004), the utility of EROD induction as a biomarker in frogs is still in question. Amphibians have been generally classified as having less catalytic activity and inducibility of MFOs than birds or mammals (Ertl and Winston 1998). A laboratory exposure using the known inducer PCB126 failed to produce increased EROD activities in leopard frogs except at concentrations > 2.3 mg/kg (Huang *et al.* 1998), whereas a recent study observed maximal EROD induction by TCDD and PCDF in *R. esculenta* primary hepatocytes (Rouhani Rankouhi *et al.* 2005). Field studies have shown that frogs accumulate persistent compounds such as PCBs but that EROD activity is not correlated with contaminant levels, especially at smaller concentrations (Huang *et al.* 1999; DeGarady and Halbrook 2003). However, a study in which *X. laevis* were exposed to field-collected water samples in the laboratory found that consistent induction of EROD activity occurred in frogs exposed to water collected downstream of industrial plants (Gauthier *et al.* 2004). The lack of consistent EROD or MROD induction in frogs in the current study indicates that these enzymes may not be sensitive biomarkers of exposure to environmental contaminants in amphibians and that the activities of the enzymes were not altered by atrazine in a way that could result in other significant changes in physiological parameters, such as circulating concentrations of hormones.

Significantly greater LSI values were observed in adult male green frogs from agricultural sites compared with nonagricultural sites. Agricultural ponds are potentially exposed to a wide variety of chemicals, including pesticides, herbicides, fungicides and fertilizers, many of which may have an effect on liver structure and function (Ertl and Winston 1998). The potential effects of these contaminants cannot be excluded as explanatory factors for the observed differences in LSI in adult male green frogs. LSI values were greater in adult male green frogs and adult female bullfrogs at site Ag2 in the fall than in early summer, which may indicate increased metabolic activity. However, this was not reflected in the measured EROD and MROD activities as would have been expected.

There was no consistent relationship observed between atrazine concentration and either EROD or MROD activities in the adult and juvenile frogs collected in this study. Specifically, no correlations between EROD activity and atrazine concentration were observed, and a statistically significant negative relationship between atrazine and MROD activity was found in adult male frogs. Atrazine was found to induce EROD activity *in vitro* at concentrations of 2.16 µg atrazine/L (Oh *et al.* 2003), a concentration similar to those measured at sites sampled in the current study. A mesocosm study with carp (*Cyprinus carpio*) found that atrazine induced P4501A1 gene expression at concentrations as small as 7 µg atrazine/L (Chang *et al.* 2005), but an earlier study of trout (*Oncorhynchus mykiss*) found no effect of 10 µg atrazine/L on EROD

activity (Egaas *et al.* 1993). A study in rats found that atrazine induced EROD activity at relatively high doses of 43 and 86 mg/kg (Hanioka *et al.* 1998). *In vitro* systems lack the complexity of whole organisms, making it difficult to extrapolate directly from cell studies to animal studies. Similarly, gene expression is only the first step in a series of regulatory pathways that result in a tissue, organ, or systemic effect in an organism. Enzyme activities in mammals have been reported to be more inducible than amphibians (Ertl and Winston 1998), and the atrazine concentrations used by Hanioka *et al.* (1998) were much greater than those measured in this study. The lack of consistent relations between atrazine concentration and EROD and MROD activities in frogs in the current study therefore may be caused by a combination of generally lower inducibilities in frogs than in other organisms and lack of atrazine-mediated induction in frogs.

The results of the bioassay for AhR activity indicated that with the exception of site Ag2, TCDD-EQ values at the study sites were < 1 pg/g dw. EROD activities in adult female green frogs from site Ag2 were significantly less than those measured at all the other sites, which was unexpected. AhR activity was detected in fractions II and III of the sediment extraction. Fraction II activity was likely caused by the presence of PAHs; site Ag2 is bordered by a dirt road, so it is possible that motor oil and other PAH-containing chemicals run off into the pond. AhR activity in fraction III was probably related to plant compounds, which tend to be labile and more easily metabolized, such that the AhR activity in this fraction likely does not represent persistent MFO induction (Jones *et al.* 2003). However, given the apparently low inducibility of EROD in frogs, the small sample size obtained for adult female frogs at site Ag2, and the potential effects of other unknown environmental factors, the cause of the significantly smaller EROD activities measured in these frogs is unlikely to have been the presence of dioxins or dioxin-like compounds and is therefore unclear.

The lack of consistent induction or suppression patterns in EROD and MROD activities observed in frogs in this study may have been caused by a variety of factors. Decreases in P4501A activities have been observed in female fish that are approaching spawning, and these activities are influenced by the health and reproductive and developmental status of fish as well as by environmental temperature (Whyte *et al.* 2000). Furthermore, it has been shown that low but long-term exposure to halogenated organic compounds such as TCDD can result in only limited induction of EROD activity in trout (Giesy *et al.* 2002). Another study of fish reported that relatively great doses of persistent halogenated organic compounds such as PCBs can inhibit the activity of CYP1A enzymes by way of competitive inhibition of enzymatic activity (Schleizinger and Stegeman 2001).

Many intrinsic and extrinsic factors can therefore influence enzymatic activity such that the presence of potential environmental inducers may not result in a sustained or large induction of activity. For example, liver size and P450 enzyme concentrations increase until metamorphosis in larval leopard frogs and stabilize once metamorphosis is complete (Khan *et al.* 1998). Although most of the juvenile frogs collected in the current study had completed metamorphosis, there may still have been differences in enzyme function and activity

between recent metamorphs and adult frogs that are unknown. Finally, it is important to note that measures of CYP1A induction in the current study were based on enzymatic end points and not on gene expression or CYP1A protein levels. Thus, the variability in the enzyme data cannot be compared with altered gene expression or protein synthesis, which can act as alternate measures of exposure to chemicals that operate through the AhR pathway. As a result, linkages cannot be made between changes in EROD activity and those genes that may be directly involved in the measured enzyme activity. Consequently, whereas there were only significant correlations between atrazine concentrations and MROD activity, this end-point has not been studied as extensively as EROD activity in amphibians, and the significance of this response is difficult to ascertain given that MROD may play a secondary role in metabolism. It is therefore not known whether induction or suppression of this enzyme has a biologically relevant effect. In addition, the sample sizes and resultant power values obtained in this study make firm conclusions difficult. The statistical power to detect differences in EROD and MROD activity between agricultural and nonagricultural sites in adult male green frogs was relatively good (0.74 and 0.68), but the power to detect differences in green frog adult female frogs was only < 0.30 and in juvenile frogs was only < 0.05 .

The results of this study indicate that the atrazine-mediated EROD induction observed *in vitro* and in other species did not occur in wild ranid frogs collected in Michigan and that correlations between atrazine concentration and MROD activity were not consistent. It is therefore unlikely that atrazine is affecting EROD and MROD in wild ranids.

Acknowledgments. We are grateful to the landowners and farmers whose cooperation made this research possible. Invaluable technical assistance was provided by Eric Higley, Sandy Mazzoni, Chris Kloss, Erika Hussar, Pamela Gabris, and Michelle Martinchek. Larry Holden provided statistical support. This research was conducted under the supervision of the Atrazine Endocrine Ecologic Risk Assessment Panel, Ecorisk, Inc, Ferndale, WA, with a grant from Syngenta Crop Protection, Inc. Additional funding for this study was provided by National Institutes of Health and Environmental Health Sciences Training Grant No. T32ES07255.

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