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dissertation entitled

ATRAZINE EXPOSURE AND REPRODUCTIVE FUNCTION OF RANID FROG SPECIES COLLECTED IN MICHIGAN

presented by

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has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Zoology and Program in Ecology, Evolutionary Biology and Behavior and Center for Integrative Toxicology

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5/2/05
Date

ATRAZINE EXPOSURE AND REPRODUCTIVE FUNCTION OF RANID FROG SPECIES COLLECTED IN MICHIGAN

By

Margaret Burkhardt Murphy

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology and
Program in Ecology, Evolutionary Biology and Behavior and
Center for Integrative Toxicology

2005

ABSTRACT

ATRAZINE EXPOSURE AND REPRODUCTIVE FUNCTION OF RANID FROG SPECIES COLLECTED IN MICHIGAN

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The triazine herbicide atrazine has been suggested to be a potential disruptor of normal sexual development in male frogs. The goals of this study were to collect native ranid frogs from agricultural and non-agricultural areas and examine gonadal gross morphological and histology, plasma steroid hormone concentrations and aromatase activity. Liver enzyme activities were also measured to determine whether atrazine exposure affected enzymes functioning in hormone metabolism.

Juvenile and adult green frogs (*Rana clamitans*), bullfrogs (*R. catesbeiana*) and leopard frogs (*R. pipiens*) were collected in the summers of 2002 and 2003 from agricultural and non-agricultural areas in south central Michigan. Atrazine concentrations were below the limit of quantification at most non-agricultural sites. Concentrations did not exceed 2 μg/L at most agricultural sites. One concentration of 250 μg atrazine/L was measured once at one site in 2002. Hermaphroditic individuals were found at a low incidence at both non-agricultural and agricultural sites in both adults and juveniles. Testicular oocytes (TOs) were found in frogs at most of the sites, with the highest incidence occurring in juvenile leopard frogs. TO incidence was significantly different between agricultural and non-agricultural sites in juveniles in 2003, and was correlated with atrazine concentrations. Neither the incidence of hermaphroditism nor TO incidence was correlated with atrazine exposure in the other adults and juveniles collected.

Plasma steroid concentrations varied among locations. Aromatase activity was measurable in less than 11% of testes in adult males, and less than 4% of testes in juvenile males. Aromatase activities were up to 2.5-fold higher in juvenile than adult females. No relationships were found between atrazine concentrations measured in water at the sites and the hormone parameters measured. LSI values in adult male frogs were significantly greater at agricultural sites. Atrazine concentrations were significantly and negatively correlated with MROD activity in adult male green frogs. LSI and EROD and MROD activities were not significantly correlated with atrazine concentrations in adult female or juvenile green frogs.

The results of this study indicate that atrazine exposure is not correlated with gross morphological or histological gonad abnormalities or with an increase in aromatase activity in male ranid frogs.

To my parents, Patrick Murphy and Patricia Burkhardt, and my sister, Cristina Murphy,
for their love and support.

ACKNOWLEDGEMENTS

The research contained in this dissertation would not have been possible without the contributions of numerous top-quality scientists and people. Support and counsel were provided by my graduate committee members, Drs. Thomas Burton and Steve Bursian, and James Harding. Dr. John P. Giesy, my graduate advisor, provided encouragement and support, as well as a blueprint for how to be a good scientist. Drs. Markus Hecker, Paul D. Jones and John L. Newsted were integral to the success of this study, providing advice and support at every turn. Drs. Katie K. Coady and Daniel L. Villeneuve an indispensable part of this research, and Eric B. Higley and Amber R. Tompsett were invaluable to the completion of both the field and laboratory aspects of this work. Louis Du Preez and Gideon J. Everson at North-West University, Potschefstroom campus, South Africa, carried out the histological sectioning and staining necessary for this project. Additional and valuable assistance for this project was provided by Sandy Mazzoni, Chris Kloss, Erika Hussar, Pamela Gabris, Michelle Martinchek, Lam Hiu Wong, Michael J. Kramer, Lacy Sharrard and John Longcore.

Funding for this project was provided by Syngenta Crop Protection, Inc., through Ecorisk, Inc. Alan Hosmer and Jim Brady of Syngenta Crop Protection, Inc. were of great help in obtaining funding and in facilitating the logistics of the study. Larry R. Holden of Sielken & Associates Consulting, Inc. provided help with statistics. Drs. Keith R. Solomon, James A. Carr, Ernest E. Smith and Glen Van Der Kraak provided helpful comments and suggestions throughout the study.

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LIST OF ABBREVIATIONS

E2	Estradiol
EROD	7-Ethoxyresorufin *O*-deethylase
GSI	Gonadosomatic index
KT	11-Ketotestosterone
LSI	Liver-somatic index
MROD	7-Methoxyresorufin *O*-deethylase
SVL	Snout-vent length
T	Testosterone
TO	Testicular oocyte

INTRODUCTION

Amphibians are a major component of many ecosystems. Worldwide declines in amphibian populations have been documented in recent years, resulting in heightened interest in researching the potential causes of these declines. Studies conducted over the past ten years have produced a variety of theories as to the causes of these declines, including habitat loss and modification, UV radiation, parasites, pathogens, pesticide exposure and/or a combination of these factors. The spread of the chytrid fungus, *Batrachochytrium dendrobatidis*, through the importation of exotic amphibian species for the pet trade and for research purposes, has become an issue of particularly great concern. However, pesticide use and impacts has also been an issue of interest, given the widespread pesticide use in the U.S. and around the world. Pesticide use is an issue of special relevance to amphibians due the fact that they often utilize ponds and wetlands adjacent to agriculture, and their breeding times in temperate climates coincide with spring pesticide applications, meaning that larval amphibians may be exposed to runoff from agricultural fields in their breeding ponds. Given the fact that the larval period is the life stage that is often the most sensitive to contaminant effects, the confluence of pesticide application and larval development period is a cause for concern.

Among high-use pesticides, the triazine herbicide atrazine has become the subject of heightened research interest in recent years. Atrazine is a pre-emergent broad leaf herbicide that was first patented for use in the U.S. in 1959. It acts by inhibiting electron transport from Photosystem II, resulting in a disruption of photosynthesis and leading to starvation in broadleaf weed species (Giddings *et al.* 2004). Because of its relatively short persistence time in the environment, atrazine became a commonly used pesticide; in

1999, approximately 31 x 10^6 kg of atrazine was used in the U.S. (U.S. EPA 2003). Due to this high usage rate and the fact that it can be transported in rainfall, atrazine is a ubiquitous environmental contaminant (Solomon *et al.* 1996). Atrazine concentrations in Midwestern reservoirs were found to average approximately 5 μg/L (Solomon *et al.* 1996), and concentrations of up to 1.8 μg atrazine/L were measured in pristine regions of Isle Royale National Park (Thurman and Cromwell 2000). Toxicity tests indicated that atrazine was not acutely toxic to frogs (Allran and Karasov 2000), but atrazine has come under greater scrutiny since 2002, when a study was published in which the authors found that atrazine at concentrations 0.1 μg/L and greater caused gonad abnormalities in male *Xenopus laevis*, the African clawed frog (Hayes *et al.* 2002). The authors stated that 0.1 μg atrazine/L was a threshold of effect, and that any concentration equal to or greater than this threshold would result in abnormal gonad development in frogs in a non-dose-dependent manner. Given the widespread use and presence of atrazine in the environment, the results of this study attracted a great deal of attention and controversy.

The mechanism of action proposed by Hayes *et al.* (2002) stated that atrazine caused gonad abnormalities in male frogs by inducing or up-regulating aromatase, the enzyme that converts testosterone to estradiol in vertebrates. They hypothesized that increased aromatase activity would result in greater estradiol concentrations in males, resulting in feminization, and producing the observed gonad abnormalities. The basis for this proposed mechanism of action was a series of *in vitro* studies which demonstrated that atrazine up-regulated aromatase activity in a human cell line (Sanderson *et al.* 1999, 2001). However, studies conducted subsequent to that of Hayes *et al.* (2002) produced inconsistent and conflicting results. Studies in which larval *X. laevis* were exposed from s

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post-hatch to metamorphosis and beyond found no significant effect of atrazine on gonad development (Carr et al. 2003, Coady et al. 2005), and a similar study on the native green frog (*Rana clamitans*) likewise found no significant atrazine effects (Coady *et al.* 2004). A field study in South Africa, where *X. laevis* is native, found inconsistent correlations between plasma steroid hormones and atrazine concentrations (Hecker *et al.* 2004), and a mesocosm study in which larval *X. laevis* were exposed to atrazine from post-hatch to six months post-metamorphosis found no significant effects of atrazine on gonad development (Jooste *et al.* 2005).

Many of the studies investigating atrazine effects were carried out using *X. laevis*, a well-known amphibian model organism. However, the life history of *X. laevis* is very different from that of native frog species in the U.S. *X. laevis* is fully aquatic in both its larval and adult life stages, and has a relatively short larval period compared to some of the ranid species that are native to the U.S., such as the green frog and bullfrog (*R. catesbeiana*), which can remain in the larval stage for up to two years (Harding 1997). In addition, although *X. laevis* has been well studied, relatively little is known about the seasonal variation in reproductive parameters such as hormones in many frog species. Seasonal changes in some hormones have been researched in the European water frog, *R. esculenta* (Fasano *et al.* 1989, Mosconi *et al.* 1994, Polzonetti-Magni *et al.* 1998), and the bullfrog (Licht *et al.* 1983), among others, but seasonal hormone patterns in many other species are not well understood. These seasonal patterns are the framework within which contaminant-mediated endocrine modulation may occur, and understanding them is therefore crucial to the interpretation of data on contaminant effects.

The goal of this doctoral research was therefore to examine whether hypothesized atrazine-induced effects were occurring in native ranid frogs in Michigan by examining gonad development, and measuring aromatase activity and plasma steroid concentrations. In addition, liver enzyme activities were measured to determine whether atrazine exposure could be correlated with changes in metabolic function.

Chapter 1

Atrazine Concentrations, Gonadal Gross Morphology and Histology in Ranid Frogs Collected from Agricultural and Non-Agricultural Areas in Michigan

Abstract

The triazine herbicide atrazine has been suggested to be a potential disruptor of normal sexual development in male frogs. The goals of this study were to collect native ranid frogs from sites in agricultural and non-agricultural areas and determine whether hypothesized atrazine effects on the gonads could be observed at the gross morphological and histological levels. Juvenile and adult green frogs (*Rana clamitans*), bullfrogs (*R. catesbeiana*) and leopard frogs (*R. pipiens*) were collected in the summers of 2002 and 2003. Atrazine concentrations were below the limit of quantification at non-agricultural sites, and concentrations did not exceed 2 μg/L at most agricultural sites. One concentration greater than 200 μg atrazine/L was measured once at one site in 2002. Hermaphroditic individuals with both male and female gonad tissue in either one or both gonads were found at a low incidence at both non-agricultural and agricultural sites, and in both adults and juveniles. Testicular oocytes (TO) were found in frogs at most of the sites, with the greatest incidence occurring in juvenile leopard frogs. TO incidence was not significantly different between agricultural and non-agricultural sites with the exception of juveniles collected in 2003. Atrazine concentrations were not significantly correlated with the incidence of hermaphroditism, but maximum atrazine concentrations were correlated with TO incidence in juvenile frogs in 2003. However, given the lack of a consistent relationship between atrazine concentrations and TO incidence, it is more

likely the TOs observed in this study result from natural processes in development rather than atrazine exposure.

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a pre-emergent herbicide first approved for use in the US in 1958, where it is used primarily on corn, sorghum and sugar cane (Solomon *et al.* 1996). Atrazine inhibits electron transport in Photosystem II, which results in a disruption of photosynthesis and in turn leads to death from starvation in broad-leaf plants (Giddings *et al.* 2004). In 1999, approximately 30.1 million kg of active ingredient were applied in the US, 75% of which was applied to corn (US EPA 2003). Between 1998 and 2002, 815 metric tons of atrazine were used in Michigan, 99.5% of which was applied to corn (Giddings *et al.* 2004).

Herbicide application generally occurs in the spring or early summer, a time which coincides with the breeding periods of many amphibian species, some of which breed in aquatic habitats that are often subject to runoff from agricultural fields. Atrazine has low volatility, but its moderate water solubility (33 mg/L at 25°C) makes it relatively mobile in soil and aquatic environments, where it tends to partition into the water column rather than sorbing to sediments (Giddings *et al.* 2004). The majority of atrazine breakdown in the environment occurs either through microbial degradation of the parent compound to the hydroxylated metabolite with loss of methyl or ethyl groups or by hydrolysis of the triazine ring (Solomon *et al.* 1996). Atrazine has been found to have a half-life in soil of from 8 to 99 d, depending on soil and environmental conditions, while the half-life of atrazine in the aquatic environment ranges from 41 to 237 d (Giddings *et* *al.* 2004). One study of atrazine biotransformation in anaerobic wetland sediment found that the half-life of the parent compound was 224 d (Chung *et al.* 1996), while a later study found that the half-life was only 38 d (Seybold *et al.* 2001). Thus, atrazine can persist in the environment, albeit at relatively small concentrations for much, if not all of the amphibian larval period. Environmental concentrations of atrazine have been reported to usually not exceed 20 μg/L, except in small temporary puddles in fields where peak concentrations can be greater than 200 μg/L for short periods of time after storm events (Solomon *et al.* 1996, Battaglin *et al.* 2000).

Exposure to agricultural chemicals, together with other factors such as habitat fragmentation, introduction of predatory species, wetland losses, UV-B radiation, and diseases have been postulated as possible causes for world-wide declines of amphibian populations (Allran and Karasov 2000 and 2001, Blaustein and Kiesecker 2002). Atrazine, while not acutely toxic to frogs at environmentally relevant concentrations (Allran and Karasov 2000, Birge *et al.* 2000, Diana *et al.* 2000, Coady *et al.* 2004), has been proposed to contribute to frog population declines because it may disrupt normal sexual development in frogs (Hayes *et al.* 2002, Hayes *et al.* 2003). The goal of the current study was to determine the incidences of testicular oocytes (TO) and hermaphroditism in ranid frogs collected from agricultural and non-agricultural areas, and to evaluate correlations between measured atrazine concentrations and these incidences.

Materials and methods

Site selection and characterization

Study sites were selected on the basis of potential atrazine exposure and the presence of relatively large populations of ranid frogs. Sites were located in three regions in south-central Michigan: Kalamazoo (KZ), the greater Lansing area (GLA) and Lapeer (LPR) (Figure 1). Sites adjacent to corn fields were classified as "agricultural", while those in the same general area, but less likely to be receiving direct runoff from corn fields, either in less agricultural areas or on private property such as backyard ponds were classified as "non-agricultural". In the first year of the study, non-agricultural sites were located in the GLA and LPR regions only; in the second year, non-agricultural sites were located in all three regions.

Water samples were collected each year from May to September to characterize the concentrations of atrazine and other agricultural chemicals. Samples were taken in 1-liter water-methanol-rinsed amber glass I-Chem bottles (Fisher Scientific, Hampton, NH, USA). All sites were sampled at least 4 times, approximately monthly from late May to September. Samples were tested for atrazine concentrations using Envirogard® triazine ELISA kits (Strategic Diagnostics, Newark, DE, USA). All samples were solid-phase extracted (SPE) using 5 ml SPE cartridges (AnSys Technologies, Palo Alto, CA, USA) to remove humic and fulvic acids prior to being used in the triazine ELISA. The method detection limit (MDL) was 0.05 μg atrazine/L, while the limit of quantification (LOQ), defined as two standard deviations greater than the MDL, was 0.17 μg atrazine/L. At each sampling event, dissolved oxygen (DO), pH, conductivity and temperature measurements were taken at four points distributed around the pond or wetland. In 2002, water samples were also tested for a number of other pesticides and heavy metals at Adpen Laboratories, Jacksonville, FL, USA. Pesticide residues were measured using EPA method 3510 (US EPA), while heavy metals were measured using ICP-MS. Water samples were tested for α-, β-, δ- and γ-BHC, hexachlorobenzene, alachlor, heptachlor, heptachlor epoxide, chlorpyrifos, 4,4-DDT, 4,4-DDD, 4,4-DDE, dieldrin, endrin, endosulfan, endosulfan sulfate, As, Cd, Cu, Pb, Ni, Se and Zn. The LOQ's for all pesticides and metals were 0.02 μg/L and 5 μg/L, respectively.

Study sites were described and categorized using the Cowardin system (Cowardin *et al.* 1979). Plant species were sampled once from each site in 2003 to determine dominant and subordinate plant types at the sites. Both aquatic and terrestrial plants were collected at each site, and only the most prevalent species present were sampled. Terrestrial plants were sampled within 2 m of the edges of ponds, or 2 m from the edges of moist wetland soils.

Frog sampling

The primary species of interest in this study was the green frog (*Rana clamitans*), which is the most common pond frog in the lower peninsula of Michigan (Harding 1997). Green frogs are territorial breeders that are associated with the aquatic environment throughout their lives and are faithful to their pond or wetland habitats (Martof 1953, Harding 1997). However, other ranid species including bullfrogs (*R. catesbeiana*) and Northern leopard frogs (*R. pipiens*), were collected as well in order to investigate interspecies differences in the measured biomarkers.

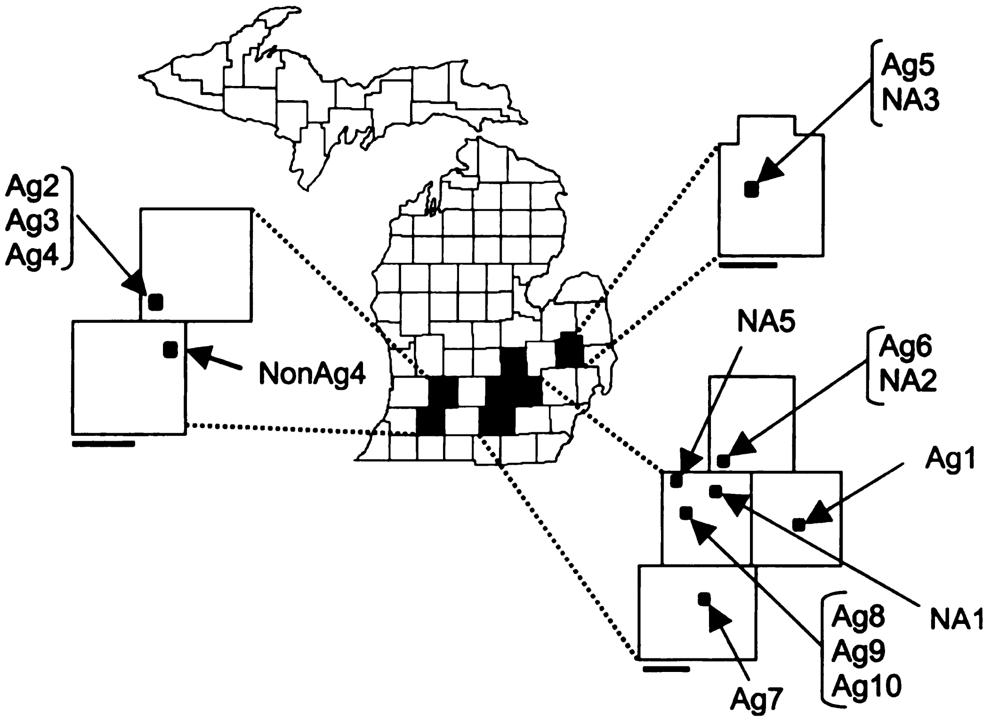

Figure 1. Site locations in Michigan. Sites were located in three major regions (left-to-right on the map): near Kalamazoo, in the greater Lansing area and near Lapeer, in both agricultural ("Ag") and non-agricultural areas ("NA"). Bars represent a distance of 20 km.

Both juvenile and adult frogs were collected in 2002 and 2003. Juveniles were collected in July in both years of the study, while adults were collected in September and October 2002, and in May and June in 2003. 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. Frogs were collected at night using hand nets and buckets. The target sample size for each sampling event was between 40 and 50 frogs per site per age class, and the minimum number collected was 17 frogs. Frogs were anesthetized in 250 mg/L MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO, USA), and visually inspected for limb, digit, eye and other malformations. Photographs were taken if malformations were observed, and each frog was weighed and its snout-vent length (SVL) was measured. All frogs were euthanized by cervical dislocation.

A longitudinal cut was made in the abdomen, and the gonads were visually inspected and photographed using a Camedia C-3040 (Olympus, Melville, NY) digital camera mounted on a model SZ40 Olympus stereomicroscope. If gonads were deemed to have normal morphology, the right gonad was removed and flash-frozen in liquid nitrogen. Gonads from adult frogs were weighed prior to freezing; while those of juveniles were too small in most cases (<0.001 g in males) to weigh accurately. The remaining gonad was left in the body and the entire frog was fixed in Bouin's solution (Sigma) for 48 h. After fixation specimens were rinsed in reverse osmosis water for 24 h, and placed in 70% ethanol for long-term storage. If gonad abnormalities were observed during gross examinations, including hermaphroditism or discontinuous gonads, the gonads were left *in situ* and the entire frog was preserved in Bouin's solution as described above. The terminology used to describe gonad anomalies in frogs has been inconsistent; for the purposes of this study, hermaphroditism was defined as an individual frog having both male and female gonad tissue in either one or both gonads.

Histology

Histological analyses were conducted on male frogs only. Gonads were removed by dissection, placed in tissue cassettes and preserved in 70% ethanol. Gonads were embedded in paraffin and serially sectioned every 7.5 μm. Sections were stained with Meyer hematoxylin and eosin and mounted on slides. Every section was examined for the presence of testicular oocytes (TO), using an Olympus BX41 TF microscope

(Olympus, Melville, NY). TOs were enumerated and reported as the number of TO per animal. A male gonad that contained more than 30% oocytes was classified as an ovotestis.

Statistical methods

Data were tested for normality using a Kolomgorov-Smirnov test with Lilliefors transformation and probability plots. Atrazine data and environmental metrics (DO, pH and temperature) were transformed using either log or square root transformation, and ANOVA and Pearson correlations were used to test for relationships with atrazine concentrations. Because limb malformation and TO incidence data were discrete, a chi-squared test was used to determine significant differences between agricultural and non-agricultural sites and relationships with atrazine concentrations were determined using Spearman correlations. All analyses were conducted using Systat 11 (SSI, Richmond, CA, USA). Significance level was set at $\alpha<0.05$ for all statistical tests.

Results

Atrazine concentrations, chemistry and vegetation

Atrazine concentrations, measured at sites designated as agricultural, ranged from less than the LOQ (0.17 μg atrazine/L) to 250 μg atrazine/L (Tables 1 and 2). The greatest concentrations were observed in 2002 at site Ag5 in the LPR region, where the landowner indicated that an 80%-atrazine formulation (Basis Gold®, DuPont, Wilmington, DE, USA) had been used. Concentrations typically peaked in the spring and early summer, although the two ponds that comprised site Ag5 showed peaks in both late

May and mid-July. Atrazine concentrations decreased from the spring maximum concentrations through the summer. At one of the Ag5 ponds, atrazine declined from a peak of 250 μg atrazine/L to less than 0.70 μg atrazine/L in 50 d. Atrazine concentrations at non-agricultural sites were all below the LOQ of 0.17 μg atrazine/L in 2002 and ranged from 0.17 to 0.23 μg atrazine/L in 2003. Mean concentrations of atrazine were significantly greater at the agricultural than the non-agricultural sites in both 2002 and 2003 (p=0.008 and p=0.010, respectively, Mann-Whitney U). Atrazine concentrations were not significantly correlated with DO, pH, temperature or conductivity in either 2002 or 2003.

Table 1. Atrazine concentrations measured in water samples collected at study sites in 2002. Concentrations were determined by triazine ELISA, for which the limit of quantification was 0.17 μg atrazine/L.

Site	Atrazine Concentration (μg/L)				
	May	June	July	August	September
NA1	< 0.17	< 0.17	< 0.17		< 0.17
NA2	< 0.17		< 0.17	< 0.17	< 0.17
NA3		< 0.17	< 0.17	< 0.17	< 0.17
Ag1	< 0.17		0.36	0.24	0.24
Ag2	< 0.17	0.52	< 0.17	< 0.17	< 0.17
Ag3	< 0.17	1.7	1.8	0.71	< 0.17
Ag4	< 0.17	< 0.17	0.48	0.17	0.11
Ag5[a]	0.83		65	2.9	< 0.17
Ag5[a]	250		0.69	< 0.17	1.9

[a] Site Ag5 was composed of two ponds, each of which had very distinct atrazine profiles. Concentrations for each pond are therefore listed separately.

None of the agricultural chemicals that were screened for were detected in 2002 water samples, and of the metals, only As and Zn were observed at concentrations greater than the LOQ of 5 μg/L. Concentrations of As did not exceed 12 μg/L, and were detected in 4 of 31 samples, 3 of which came from one of the ponds at Ag5. Zn was detected in 19 of 31 samples, but only 3 of these samples had concentrations that exceeded 20 µg/L. However, Zn was detectable at all sites at least once throughout the summer, with the highest number of detections occurring in samples from site Ag2 (6 of 19 detections).

Most of the study sites were well vegetated with the exception of Ag1, which was a backyard pond with very little surrounding and emergent vegetation. All other sites were characterized by a variety of plant species, the most common of which were cattails (*Typha angustifolia*, *T. latifolia*), bulrushes (*Scirpus acutus*), and various grass and sedge species. Further detail on plant types and other site characteristics can be found in Appendix A.

Table 2. Atrazine concentrations measured in water samples collected at study sites in 2002. Concentrations were determined by triazine ELISA as described above.

Site	Atrazine Concentration (µg/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		< 0.17	< 0.17	< 0.17	< 0.17
Ag2	0.20	< 0.17	< 0.17	< 0.17	< 0.17
Ag3[a]	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		0.70	0.73	0.63	0.45
Ag10		0.21	0.33	< 0.17	< 0.17

[a] Site Ag-3 dried up in late summer.

Limb malformations

Limb and digit malformations were observed at 4 of 8 sites in 2002 and at 5 of 10 sites in 2003 (Table 3). Individuals with malformations were all green frogs. The most commonly observed malformations were fused digits (syndactyly), missing digits (ectrodactyly), extra digits (polydactyly) and missing limbs (ectromelia). Missing eyes were found in one juvenile in 2002 and in one adult and one juvenile in 2003. One adult from Ag7 had entirely black eyes. No frogs with extra limbs were collected. The incidence of malformation was not significantly different between agricultural and non-agricultural sites in juveniles in 2002 (p>0.995, df=8, χ^2 test) or 2003 (p>0.950, df=10 and p>0.500, df=2, respectively, χ^2 test). Statistical comparisons could not be made between agricultural and non-agricultural sites for adults in 2002 due to a 0% incidence of limb malformations at the non-agricultural sites.

Gross gonadal morphology

The site incidence of hermaphroditism was less than 5% at both agricultural and non-agricultural sites in both years of the study. Frogs exhibiting hermaphroditism were found at only three of the fifteen study sites, and represented an overall incidence of 0.54% in 2002 and 0.29% in 2003. Hermaphroditic individuals were all green frogs (Figures 2 and 3). In 2002, one juvenile hermaphrodite was found at each of sites NA3 and Ag4 (2.1% and 2.0% incidence, respectively). The juvenile from site NA3 had a normal testis on one side and an ovotestis on the other (Figure 3E), while the juvenile from site Ag4 had a complete testis on one side and a complete ovary on the other (Figure 2C, 3D). No hermaphroditic adults were collected in 2002. In 2003, one adult

Table 3. Limb malformation rates in green frogs (*Rana clamitans*).

2002					2003				
Site	Adult n	Malformation Rate (%)	Juvenile n	Malformation Rate (%)	Site	Adult n	Malformation Rate (%)	Juvenile n	Malformation Rate (%)
NA1	48	0	55	0.0	NA1	22	0.0	45	6.7
NA2	45	0	36	0.0	NA4	14	14.3	42	4.8
NA3	46	0	48	4.2	NA5	44	0.0	52	0.0
Ag1	5	0	17	0.0	Ag2	64	0.0	47	4.3
Ag2	17	5.9	95	1.1	Ag3	16	0.0	0[b]	0.0
Ag3	30	0	42	4.8	Ag6	22	0.0	0[c]	0.0
Ag4	51	0	49	0.0	Ag7	29	13.8	0[d]	0.0
Ag5	16	12.5[a]	23	0.0	Ag8	41	2.4	0[d]	0.0
					Ag9	43	4.7	47	2.1
					Ag10	27	0.0	0[d]	0.0

[a] The two malformations found at site Ag 5 were found in the same frog. The malformation rate based on individuals at this site is therefore 6.25%. [b] Juveniles could not be collected at this site because it had dried up by mid-summer. [c] Only juvenile leopard frogs were collected at this site. [d] Juveniles were not found at this site.

hermaphrodite was collected from site Ag7 (3.4% incidence) (Figure 2D, 3E). This frog had male secondary sexual characteristics, including an enlarged tympanum and yellow coloration in the throat area. The frog had small lumps of ovarian tissue located both above and below the testis, and small lumps of testicular tissue located above and below the ovary. None of the green frog or leopard frog juveniles collected in 2003 were hermaphrodites.

Discontinuous gonads were found only in one adult green frog from site NA2 in 2002 (3.3% incidence), and in two juvenile male frogs collected at site NA5 in 2003 (7.1% incidence). A male bullfrog with only one testis was collected from site Ag5 in 2002.

Table 4. Sample size, number of frogs with testicular oocytes (TO), number of TO per animal and the species in which TO were found based on histological examination of the gonads in males in 2002.

Site	Adult n	# Frogs with TO	#TO per Animal	Species	Juvenile n	# Frogs with TO	#TO per Animal	Species
NonAg 1	38	2	1	R. clamitans	31	1	1	R. clamitans
NonAg 2	30	2	2, 15	R. clamitans	22	0	0	R. clamitans
NonAg 2	8	2	1, 2	R. pipiens	4	2	1, 8	R. pipiens
NonAg 3[b]	28	5	1, 1, 1, 3, 27	R. clamitans	23	1	5	R. clamitans
Ag 1	10	0	n/a	R. clamitans	4	0	0	R. clamitans
Ag 2	13	0	n/a	R. clamitans	44	4	1, 1, 4, 12	R. clamitans
Ag 2	13	1	95	R. catesbeiana	-	-	-	-
Ag 3	13	2	3, 32	R. clamitans	25	4	1, 1, 1, 7	R. clamitans
Ag 3	6	0	n/a	R. catesbeiana	-	-	-	-
Ag 4[b]	29	8	1, 2, 2, 4, 5, 6, 7, 33	R. clamitans	24	2	1, 11	R. clamitans
Ag 5	7	1	1	R. clamitans	14	1	1	R. clamitans
Ag 5	2	1	76	R. catesbeiana[a]	-	-	-	-

[a] This frog had only one testis. [b] One juvenile of mixed sex was collected at this site.

Table 5. Sample size (n), number of frogs with testicular oocytes (TO), number of TO per animal and species in which TO were found based on histological examination of the gonads in males in 2003.

Site	Adult n	# Frogs with TO	#TO per Animal	Species	Juvenile n	# Frogs with TO	#TO per Animal	Species
NA1	16	1	81	*R. clamitans*	20	0	n/a	*R. clamitans*
NA4	12	1	1	*R. clamitans*	24	1	3	*R. clamitans*
NA4	12	0	n/a	*R. catesbeiana*	-	-	-	
NA5	33	3	1, 4, 4	*R. clamitans*	27	3	4, 5, 8	*R. clamitans*
Ag2	37	3	1, 1, 2	*R. clamitans*	19	3	1	*R. clamitans*
Ag2	7	0	n/a	*R. catesbeiana*	-	-	-	-
Ag3	7	1	1	*R. clamitans*	-	-	-	-
Ag3	6	0	n/a	*R. catesbeiana*	-	-	-	-
Ag6	15	2	6, 7	*R. clamitans*	27	22	1, 1, 3, 3, 5, 7, 9, 9, 10, 11, 12, 12, 15, 21, 26, 30, 31, 45, 48, 57, 69, 148	*R. pipiens*
Ag6	3	0	n/a	*R. pipiens*	-	-	-	-
Ag7[a]	22	3	1, 1, 1	*R. clamitans*	-	-	-	-
Ag8	14	1	2	*R. clamitans*	-	-	-	-
Ag9	32	5	1, 1, 2, 3, 5	*R. clamitans*	22	6	1, 1, 2, 2, 2, 6	*R. clamitans*
Ag10	24	2	1, 5	*R. clamitans*	-	-	-	-

[a] One adult of mixed sex was collected from this site.

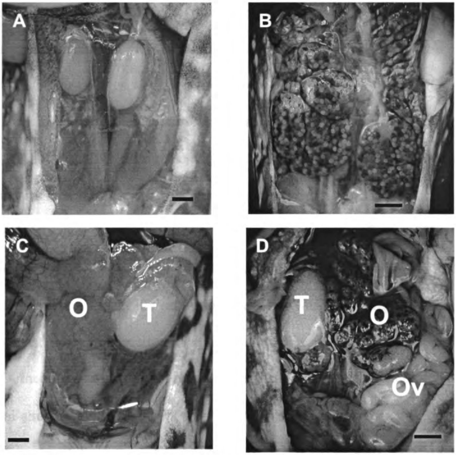

Figure 2. Gonadal gross morphology of ranid frogs. (A) Juvenile male with normal morphology. Note the slight difference in gonad size, with the right larger than the left; this morphology appears to be typical in ranids. Bar represents 2 mm. (B) Adult female with normal morphology; bar represents 5 mm. (C) Hermaphroditic juvenile green frog, having both an ovary (O) and a testis (T); bar represents 2 mm. (D) Hermaphroditic adult green frog, having testis (T), ovary (O) and oviduct (Ov); bar represents 5 mm.

Gonad histology

Testicular oocytes (TO) were observed in both juvenile and adult males of all three ranid species collected in both 2002 and 2003 from most of the ponds studied (Figures 3 and 4). TOs were discrete structures separate from the surrounding testicular tissue (Figure 3A inset), and consistently had the morphology of a previtellogenic (stage

II) oocyte (Dumont 1972). The number of TO per animal was variable, ranging from 1 or 2 per animal to a maximum of 148 observed in a juvenile leopard frog (Tables 4 and 5). Four extra-testicular oocytes were observed in one juvenile green frog from site NA5 in 2003 (Figure 3B). Juvenile leopard frogs had the greatest overall incidence of TO (Figure 3C, Table 5). The juvenile hermaphrodite from site NA3 was found to have both oocytes and regions of testicular tissue in the same lobed tissue mass, although a large portion of the tissue appeared undifferentiated or indeterminate (Figure 3E). The adult hermaphrodite collected at site Ag7 in 2003 was found to have both stage II and stage III oocytes in its ovarian tissue, indicating that this frog was beginning its breeding cycle, and was at least a partially functional female (Figure 3F).

TO incidence was not significantly different between agricultural and non-agricultural sites in adults ($p>0.995$, df=8, χ^2 test) or juveniles ($p>0.100$, df=8, χ^2 test) from 2002, or in adults collected in 2003 ($p>0.995$, df=12, χ^2 test). TO incidence was significantly greater at agricultural sites than at non-agricultural sites in juveniles collected in 2003 ($p<0.005$, df=2, χ^2 test), and was correlated with maximum atrazine concentrations (Spearman R=0.820).

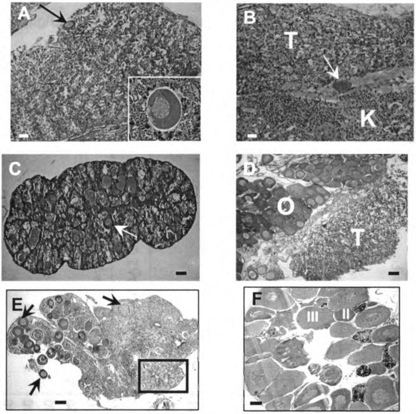

Figure 3. Gonad histology of ranid frogs. (A) Green frog testis with a single oocyte (arrow). Inset shows typical stage II oocyte morphology. Bar represents 50 μm. (B) Green frog with an extra-testicular oocyte lying between the testis (T) and kidney (K) (arrow). Bar represents 50 μm. (C) A leopard frog testis with multiple oocytes (arrow). This frog had a total of 57 oocytes in the testis. Bar represents 100 μm. (D) Juvenile green frog (see Figure 3C) having both ovarian (O) and testicular tissue (T). Bar represents 100 μm. (E) Ovotestis in a juvenile green frog showing oocytes (arrows), and a region of testicular tissue (box) within a larger region of undifferentiated tissue. This frog also had a normal testis. Bar represents 100 μm. (F) Oocytes from the hermaphroditic adult from site Ag7 (see Figure 3D). Note that this frog has both stage II and stage III oocytes. Bar represents 100 μm.

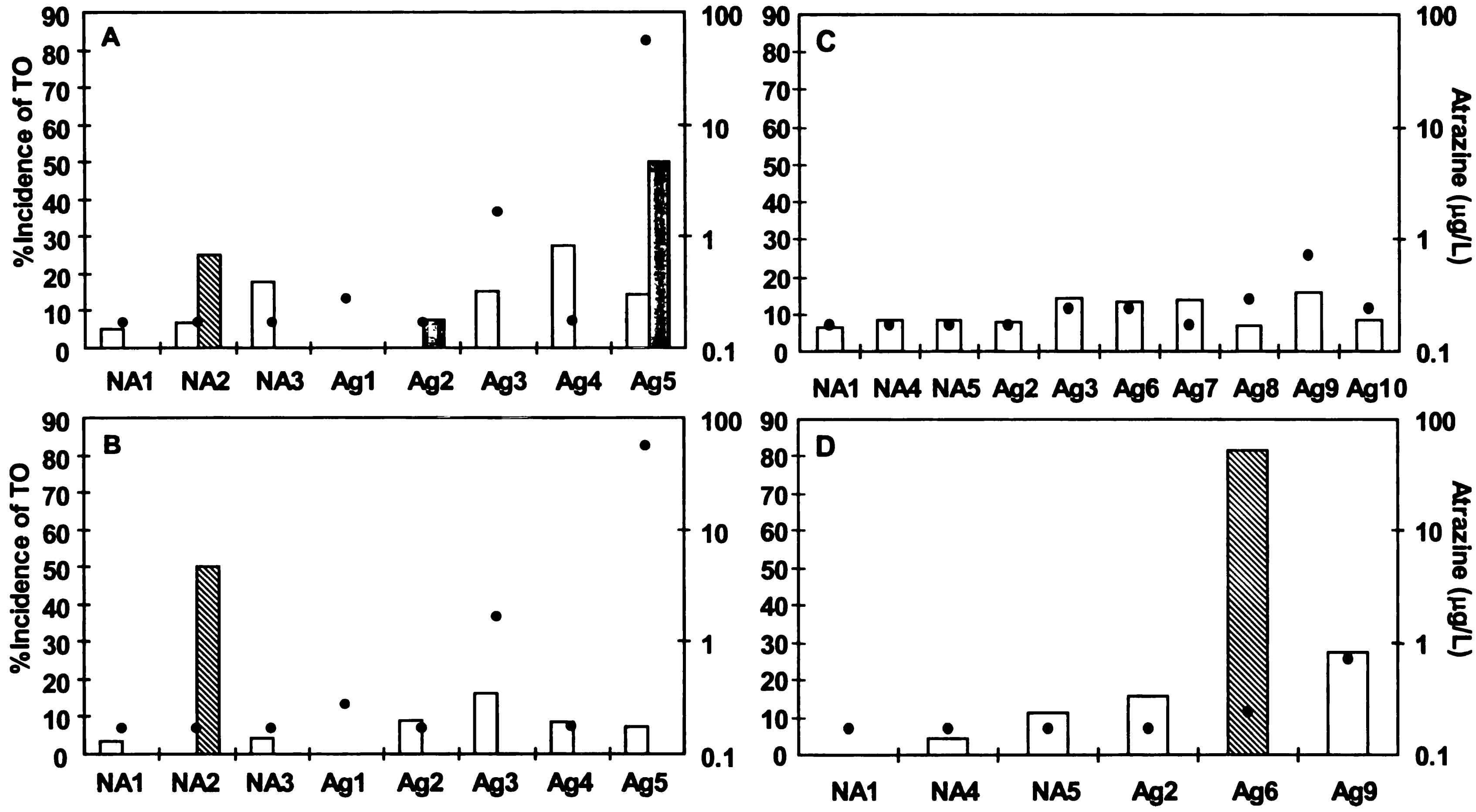

Figure 4. Incidence of testicular oocytes in adult and juvenile ranid frogs. Incidences were determined in adults (A) and juveniles (B) collected in 2002 and in adults (C) and juveniles (D) collected in 2003. *R. clamitans* are represented by the open bars, *R. catesbeiana* are represented by the shaded bars, and *R. pipiens* are represented by the crosshatched bars. Points represent the 75% quartile of the atrazine concentrations measured at the sites.

Discussion

Atrazine concentrations measured at study sites generally did not exceed 2 μg atrazine/L, with the exception of the two ponds that were sampled at site Ag5 in 2002. Rain fell in the area of these sites one week previous to water collection, which likely resulted in runoff into the ponds from recent spraying. The two ponds at Ag5 did not show simultaneous atrazine peaks—one peaked at 250 μg/L in May, while the other peaked at a lesser concentration of 65 μg atrazine/L in June. These different peaks probably represent separate application regimes for the two fields. The pond that peaked in May was at the base of a sloped field next to a dirt road, while the other pond was located in the middle of a flat field. These topographical differences likely resulted in a greater amount of runoff in the pond at the base of the sloped field, and resulted in the higher concentration measured in the pond. However, it is possible that both ponds reached the same peak concentrations, but that these were not captured equally by sampling water on a monthly basis. The combination of formulation type, topography and differences in vegetation at the other sites sampled in 2002 and 2003 likely contributed to the smaller atrazine concentrations measured in these ponds. The relatively rapid degradation rate of atrazine that was observed at the Ag5 ponds is similar to that of 38 d (Seybold *et al.* 2001) and 48 d (Moore *et al.* 2000) seen in laboratory experiments with anaerobic wetland sediment, indicating that atrazine does not persist in dynamic systems such as wetlands.

Both As and Zn were measured in water samples collected in 2002, but the effects of these metals on amphibians have not been well studied. Zn was found to inhibit glucose-6-phosphate dehydrogenase (G6PDH) activity in female *Bufo arenarum* at concentrations as small as 4 µg/L (Naab *et al.* 2001, de Schroeder 2005), and inhibited sperm motility in *Xenopus laevis* by approximately 12% at concentrations of 31 µg/L (Christensen *et al.* 2004). In this study, all 19 samples with detectable levels of Zn had concentrations greater than 4 µg Zn/L, but only three samples from sites Ag2 and Ag3 had concentrations greater than 31 µg Zn/L (34 and 41 µg Zn/L at site Ag2 and 53 µg Zn/L at site Ag3). Inhibition of G6PDH can result in a decrease in the production of reducing agents, which can have wide-ranging effects on metabolic function, including enzymes that function in reproduction. Reproductive effects such as reduced sperm motility could be indicative of other effects on the endpoints of interest in this study. However, since these specific biomarkers and endpoints were not measured, Zn effects on the species collected in this study are unknown.

Concentrations of As were near the national drinking water standard of 10 µg As/L set by US EPA (US EPA 2002), but only one sample from site Ag5 had a concentration greater than this standard (12 µg As/L). The effect of As in amphibians has also not been well studied, with most of the current information coming from accumulation studies at sites contaminated with metals. A study examining concentrations of trace elements in *B. terrestris* inhabiting coal ash settling basins found arsenic and zinc concentrations of approximately 0.23 µg As/g and 200 µg Zn/g, respectively, in toads from the reference site, but the study did not examine effects of these concentrations (Hopkins *et al.* 1998). Sediment concentrations were not measured in the current study, making direct comparisons to accumulation studies difficult. The effects of the concentrations of Zn and As measured at the study sites are therefore unknown.

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The frogs collected in the course of this study exhibited a great deal of variation in reproductive stage. Collection of adult frogs took place late in the summer in 2002, when it was presumed that all individuals would be at the end of their breeding cycle. However, due to the long breeding season in green frogs (May-July) and the fact that they may breed more than one time per summer, this scheme was found to be insufficient in terms of reducing variability. Females collected in 2002 ranged from having abdomens full of eggs to having eggs that were almost completely reabsorbed. In 2003, adults were collected in May, in the hopes that collecting prior to or early in the breeding season would reduce the variability in reproductive stage among individuals, but the variability observed in 2003 was similar to that seen in 2002.

Green frog tadpoles over-winter in sediments if they do not metamorphose before the end of the summer. Typically, tadpoles that hatch late in the breeding season will over-winter, while those that hatch earlier in the summer will metamorphose (Harding 1997). Whether a juvenile is from the previous or current year is readily apparent based on body size, such that it was possible to collect juveniles from the current year with a high degree of certainty that they were in fact from egg masses laid earlier that summer. One study found that green frog over-wintering tadpoles and tadpoles that hatched in the same year were of approximately equal size at metamorphosis (Martof 1956). However, juveniles that were deemed second-year or over-wintering frogs in this study were found to be very different in their overall size. In general terms, the ratio of weight-to-snout-vent-length (SVL) in over-wintering frogs was in the range of 1.9-2.9 while that of same-year juveniles was 0.6-1.6. This relationship applied only to juveniles that had completed tail resorption, and there was some site-to-site variation, with tadpoles metamorphing at larger sizes at some sites.

The issue of limb malformations in frogs has attracted a great deal of interest in recent years due to the observation of severely malformed frogs in Minnesota in 1995 (Souder 2000). Highly variable malformation rates were found in green frogs and bullfrogs collected from agricultural sites and non-agricultural sites in Canada, but the site types were not significantly different in terms of malformation rates (Ouellet *et al.* 1997). A study on green frogs and bullfrogs in New Hampshire found that the rate of malformation per site ranged from 0 to 9.3% (Sower *et al.* 2000), which is similar to the range observed in this study. Many hypotheses have been proposed to explain the occurrence of limb malformations in amphibians, among them UV radiation, parasites, and contaminants; of these, the strongest evidence appears to support parasites as the causative agent of malformations, although more research is needed (Ankley *et al.* 2004). One study found that exposure to both atrazine and parasites increased the proportion of limb malformations in wood frogs (*R. sylvatica*) (Kiesecker 2002), but given the fact that agricultural and non-agricultural sites were not significantly different in terms of malformation rate in the current study, it is unlikely that atrazine contributed to the occurrence of limb malformations.

The major question surrounding atrazine is whether it affects sexual development in frogs, but answering this question is complicated by the fact that there are aspects of sexual development in some species of frogs that are not well understood. Natural history studies have indicated that the presence of TOs occurs in male frogs at some background level of incidence (Witschi 1921). More recent studies of genetics and ontology have shown that amphibians in general, and frogs in particular, possess a great deal of plasticity when it comes to sexual development patterns, and that these may change in response to environmental conditions (Wallace *et al.* 1999). Sex races with distinct patterns of sexual development have been observed in European grass frogs (*R. temporaria*) (Witschi 1930), bullfrogs (*R. catesbeiana*) (Hsu and Liang 1970) and bi-colored frogs (*R. curtipes*) (Gramapurohit *et al.* 2000).

The ability to discuss incidences of gonadal anomalies and/or abnormalities and make accurate comparisons among studies requires a common terminology. One of the limitations in attempting to interpret both gonadal gross morphology and histology data is that authors often do not distinguish between the various degrees of intersex and use terms interchangeably. For example, the terms "hermaphrodite", "intersex" and "mixed sex" are all used to describe the condition where ovarian and testicular tissue are mixed in the same gonad, but "hermaphrodite" and "intersex" are also used to describe ovarian and testicular tissue that is segregated laterally or rostrally/caudally in the same animal (e.g. Hayes et al. 2002, Carr et al. 2003, Coady et al. 2004). "Hermaphrodite" has also been used historically as a catchall term for any sort of gross gonadal abnormality (Witschi 1921, 1930). The term "sex-reversal" has also been used to describe frogs with TO (Hayes et al. 2003). Use of this term is appropriate in the context of laboratory studies when frogs are exposed to chemicals and the treatment groups differ from the 50:50 male:female sex ratio found in controls (e.g. MacKenzie et al. 2003), but it is difficult to have full knowledge of processes that could cause sex reversal in wild-caught organisms. The study of endocrine disruption in frogs specifically and amphibians in general would be therefore greatly improved by some degree of consensus on how to describe and categorize reproductive effects at the histological level. Research in fish has produced classification systems for gonad abnormalities, such as the intersex index that has been developed for roach (*Rutilus rutilus*) (Jobling *et al.* 1998) and the ovotestis severity index (OSI) that has recently been developed in European flounder (Bateman *et al.* 2004), but a similar index has not been developed for frogs. As discussed previously, interspecies differences can greatly complicate matters, but given the state of amphibian populations around the world and the need for accurate assessments of individual and population health, consensus building seems of the utmost importance.

The occurrence of hermaphroditic individuals has historically been observed in healthy frog populations and is unlikely to be the result of exposure to atrazine as suggested by Hayes *et al.* (2002, 2003). In the early 1920s and 1930s, Witschi reported his observations of hermaphroditism in larval European common frogs (*R. temporaria*) (1921), later finding an 8% incidence of hermaphroditism in adult *R. temporaria* (1930). He concluded that such low incidences of hermaphroditism appeared to be a natural component of some European common frog populations (Witschi 1930). However, Witschi did not indicate whether this hermaphroditism was on the order of TO or on the order of fully organized tissue. In this context, the low number of hermaphroditic individuals collected in this study therefore likely represents background incidence rates as opposed to a contaminant effect.

The single adult hermaphrodite that was collected at site Ag7 presents an interesting case study. This frog had fully mature gonads of both types and male secondary sex characteristics. This frog also demonstrated a more advanced stage of oocyte development, having both stage II (pre-vitellogenic) and stage III (early vitellogenic) oocytes when compared to other individuals with TOs. This frog was found to have relatively small plasma concentrations of estradiol (E2) (0.25 ng E2/ml) and very great concentrations of testosterone (T) (34.73 ng T/ml) (see Chapter 2). Conversion of T to E2 by the cytochrome p450 enzyme aromatase in the female gonad would result in normal seasonal maturation of the ovary, while testosterone would be converted to dihydrotestosterone and other androgens in the testis and would also trigger the development of the observed male secondary sex characteristics. However, the presence of vitellogenic oocytes in the ovary is difficult to explain, given that vitellogenin production in the liver is stimulated by high plasma E2 concentrations, which were not observed in this frog. The exact mechanism of ooycte development and maturation in this frog is therefore unclear at this stage.

The occurrence of TOs in frogs has been the focus of a great deal of interest and debate. The TOs observed in this study were consistent with the description of pre-vitellogenic stage II of oocyte development as given by Dumont (1972). TO incidence was not significantly different between agricultural and nonagricultural sites, and TOs were observed in all three species collected and in both adults and juveniles. Atrazine concentrations were correlated with TO incidence in juvenile frogs collected in 2003, but not in adult frogs in either year or in juveniles in 2002. This inconsistent result indicates that the incidence of TOs in these species is likely not related to atrazine exposure, including the relatively great concentrations measured during at least part of the tadpole development period in 2002. This result is in contrast to the studies of Hayes *et al.* that suggest that concentrations as small as 0.1 μg atrazine/L can induce gonadal abnormalities, such as discontinuous testes, TOs and hermaphroditism in the African clawe

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clawed frog (*X. laevis*) (Hayes *et al.* 2002). The occurrence of TOs in frogs in that study was indicated to be caused by exposure to atrazine and was never observed in unexposed frogs (Hayes *et al.* 2002). A field study of leopard frogs (*Rana pipiens*) conducted in 2002 found TO in 92% of the males in one population collected from a reference pond in Wyoming (Hayes *et al.* 2003), but no TO were observed at that site in subsequent years. In contrast, one study frequently cited to support the contention that atrazine affects development in frogs is a field study on cricket frogs (*Acris crepitans*) (Reeder *et al.* 1998). While Reeder *et al.* found a significant correlation between intersex animals— a category in which the authors included both hermaphroditic animals as well as those having TOs—and PCB and PCDF contamination, they found no significant correlation of atrazine concentrations with gonadal anomalies. Another study by the same authors on cricket frogs using museum specimens found that the historical incidence of intersex was greatest between 1946 and 1959 when PCB and DDT use was greatest, and before atrazine was approved for use, and that this incidence has declined sharply since this period (Reeder *et al.* 2005).

The TOs observed in this study appear to be of the same morphology that has been seen in other studies in which frogs were exposed to atrazine (Carr *et al.* 2003, Hayes *et al.* 2003, Coady *et al.* 2004, Jooste *et al.* 2005). Laboratory exposures of *X. laevis* to atrazine have caused either no significant atrazine effects on the incidence of TOs or the occurrence of hermaphroditism (Coady *et al.* 2005), or induced effects only at 25 µg atrazine/L (Carr *et al.* 2003), while a laboratory exposure of green frog tadpoles to atrazine found TO in control frogs at an incidence of 12% (Coady *et al.* 2004). A field study with *X. laevis* conducted in South Africa did not find any significant effects of

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atrazine exposure on testicular or laryngeal development at concentrations smaller than 10 µg atrazine/L (Smith *et al.* 2003). Furthermore, no effects of atrazine on gonadal development were observed in mesoocosms where *X. laevis* were exposed to atrazine concentrations of 0, 1, 10, and 25 µg atrazine/L (Jooste *et al.* 2005). In fact, it was observed by Jooste *et al.* that, based on histological examination of testes of recently metamorphosed frogs, 57% of the reference group animals had TOs compared to 57%, 59%, and 39% of the 1, 10 and 25 µg/L atrazine groups, respectively. The number of TO per individual varied from 0 to 58 with means of 9.5, 9.8, 8.5, and 11.1 for the 0, 1, 10 and 25 µg atrazine /L groups, respectively. In addition, these authors found that the number of TOs per animal decreased as the frogs grew. Males that were analyzed 10 months post-metamorphosis had a reduced number of TOs per animal when compared to males analyzed at metamorphosis. The authors hypothesized that the presence of TO is a normal part of development, and that TOs were broken down and reabsorbed over time. This pattern does not seem to hold for green frogs, since juvenile and adult frogs had numbers of TOs in the same range, but it may occur in leopard frogs.

The juvenile leopard frogs collected in this study were found to have a 4-to-20-fold greater incidence of TOs than either green frogs or bullfrogs. This greater incidence is consistent with that observed in another field study, where an incidence of 92% was observed in a population of leopard frogs (Hayes *et al.* 2003). The interspecies disparity may be due to several factors, including a different development pattern in leopard frogs that results in a greater occurrence of TOs, greater sensitivity on the part of leopard frogs to environmental contaminants or other ecological stressors, or a combination of these factors. The results of a laboratory exposure of leopard and wood frog tadpoles to several endocrine-disrupting compounds in which both species developed oocytes at varying incidences indicated that leopard frogs were more sensitive than wood frogs to the effects of these compounds (MacKenzie *et al.* 2004), but further research into this issue is needed. Adult leopard frogs collected for this study had no more than 2 TOs per animal, but because they were encountered infrequently, only 11 adults were collected over two years. It is possible that the resorption hypothesis proposed by Jooste *et al.* (2005) applies to leopard frogs as well, but more research is needed to determine if this is the case.

TOs were not found at some sites in this study, which may be due to an insufficient sample size or to population structures such as sex races that are not currently understood. Distinct development patterns have been found in populations of *R. catesbeiana* in Taiwan, with some populations passing though a bisexual or hermaphroditic stage before the differentiation of tissues into ovaries and testes, while in others ovaries developed in all frogs before the males then developed testes after metamorphosis (Hsu and Liang 1970). Another study reported that *R. curtipes* tadpoles followed the latter pattern, with testicular development in males accompanied by oocyte degradation (Gramapurohit *et al.* 2000). The TOs observed in this study may therefore be developmental remnants that did not fully degrade and persisted into sexual maturity. This explanation has been hypothesized by Jooste *et al.* (2005), and is supported by the fact that most frogs collected in the current study, with the exception of leopard frogs, had fewer than 10 TOs. In 2002, 33% of adult frogs and 13% of juveniles with TOs had more than 10, and in 2003, neither adults nor juveniles had more than 10 TOs. It is possible, therefore, that within the percentage of male green frogs with TOs, most of these result from incomplete differentiation, while the remainder may represent a more severe disruption of normal development. However, the diversity of development patterns employed by amphibians complicates attempts to assess these patterns because "normal" or "background" conditions are currently poorly understood. The 57% incidence of TOs found in reference *X. laevis* by Jooste *et al.* (2005), a much higher incidence than was seen in frogs in this study with the exception of leopard frogs, provides further evidence of the need for more understanding of background incidence rates. Similarly, TOs were found in bullfrogs, but these occurred only in two animals and at relatively high numbers per animal (76 and 95). The majority of bullfrogs collected had no TOs, which may indicate that they are of the bisexual race described by Hsu and Liang (1970). As with leopard frogs, however, far fewer bullfrogs than green frogs were collected, making it difficult to draw definitive conclusions.

While the effects of TO occurrence at the population level are not known, it is unlikely that the small incidence of TOs in individual frogs would result in decreased reproductive fitness. A study in fish exposed to endocrine-disrupting compounds in the wild found that reproductive fitness in males, as measured by sperm characteristics and reproductive success, was reduced in severely feminized fish from contaminated areas compared to fish from control areas (Jobling *et al.* 2002). The authors used four categories ranging in severity from the presence of an ovarian cavity to distinct regions of ovarian tissue to describe intersex individuals. However, to date the effects of TOs on reproduction in frogs has not been researched. In our study, we observed a low-level incidence of gonadal abnormalities and TOs across all locations with no apparent relationship to atrazine exposure, including some relatively great concentrations for part of the larval period. Given the generally low incidence of hermaphroditism and the lack of correlation of TO incidence with agricultural areas or sites with elevated atrazine concentrations, it is unlikely that the exposure to atrazine observed in this study resulted in deleterious effects on gonadal structure or function.

The number of individuals collect at some locations was small. Power analyses indicated that 120 individuals per sex per site would need to be collected in order to be able to distinguish between TO incidences of 1 and 10% at β=0.8. While this sample size is one that can be achieved at some sites, some of the ponds that were used for this study were not able to support populations of this size. The target of 40-50 frogs was reached at most sites, but some of the agricultural sites did not have enough of a population to reach this target and still sample responsibly. Agriculture often requires land modification that can alter crucial habitat, resulting in the loss of the metapopulation structure that many amphibian species, especially highly aquatic species such as green frogs, rely on for the recolonization of habitats that may be lost in dry years.

The results of this study indicate that hermaphroditism and testicular oocytes occur at some background incidence level in ranid frogs, and that these incidences are unrelated to atrazine exposure. Species-specific differences in testicular ooctye incidence among the three ranid species collected in this study indicate that further research on development patterns and normal background levels of these gonad anomalies is needed.

Chapter 2

Plasma Steroid Hormone Concentrations, Aromatase Activities and GSI in Ranid Frogs Collected from Agricultural and Non-Agricultural Sites in Michigan (USA)

Abstract

The triazine herbicide atrazine has been hypothesized to disrupt sexual development in frogs by up-regulating aromatase activity, resulting in greater estradiol (E2) concentrations and causing feminization in males. The goal of this study was to collect native ranid frogs from atrazine-exposed ponds and determine whether relationships could be determined between measured atrazine concentrations and the gonadosomatic index (GSI), plasma concentrations of testosterone (T), E2 or 11-ketotestosterone (KT), or with aromatase activity. In the summers of 2002 and 2003, 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. Atrazine concentrations were below the limit of quantification at non-agricultural sites. Atrazine concentrations did not exceed 2 μg/L at most sites, but a concentration of 250 μg atrazine/L was measured once at one site in 2002. Plasma steroid concentrations varied among locations. Aromatase activity was measureable in less than 11% of testes in adult males, and less than 4% of testes in juvenile males. Median aromatase activities in ovaries of adult females ranged from 3 to 245 fmol/h/mg protein, and maximum activities were 2.5-fold greater in juveniles than in adults. Atrazine concentrations were significantly and positively correlated with KT in adult females in 2002 and with T in juvenile males in 2003, but

were not significantly correlated with any other parameter. These results indicate that atrazine does not up-regulate aromatase in green frogs in the wild, and does not appear to have a clear-cut effect on plasma steroid hormone concentrations.

Introduction

The triazine herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is the subject of heightened research interest recently because of its hypothesized effects on sexual development in male frogs (Hayes *et al.* 2002, 2003). Atrazine is applied to crops including corn, sorghum and sugar cane; an estimated average of 25.2 to 29.9 x 10^6 kg is applied to fields in the U.S. per year (US EPA 2003). Application may occur either pre- or post-emergence, although most applications occur during the pre-emergence period in April or May. Concentrations of atrazine can reach maximum values of approximately 100 μg/L in streams and rivers in agricultural areas after storm events (Solomon *et al.* 1996, Giddings *et al.* 2004). However, these maximum concentrations have a very short duration and longer-term moving average concentrations seldom exceed 20 ug/L (Solomon *et al.* 1996, Giddings *et al.* 2004). Elevated concentrations of atrazine are observed in the spring and can coincide with the breeding periods of many amphibian species that utilize farm ponds, wetlands and other habitats often exposed to runoff from agricultural fields. This fact, coupled with widespread declines in amphibian populations, has prompted concern about the potential effects of agricultural chemicals, including atrazine.

Several studies have been conducted in the past few years to address the question of whether atrazine has effects on development and/or reproductive function in frogs.

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Studies conducted under laboratory conditions in which the African clawed frog (*Xenopus laevis*), an amphibian model, has been exposed to atrazine from post-hatch through metamorphosis and beyond have produced conflicting results. One study found that atrazine increased the incidence of gonadal abnormalities at concentrations as small as 0.1 μg/L (Hayes *et al.* 2002). However later studies using the same range of concentrations did not find significant effects of atrazine on gonad development of juvenile male *X. laevis* (Carr *et al.* 2002, Coady *et al.* 2005) or *R. clamitans* (Coady *et al.* 2004). However, estradiol concentrations in male *X. laevis* were significantly different from the control in the 1 μg atrazine/L treatment (Coady *et al.* 2005).

Atrazine has been postulated to affect the development of frog gonads by up-regulation of the cytochrome p450-requiring enzyme aromatase (*CYP19*). This enzyme is responsible for converting testosterone to estradiol. It has been suggested that atrazine, by up-regulating aromatase expression, can result in an increase in estradiol concentrations with a concomitant decrease in plasma concentrations of testosterone (T), which can result in both feminization and demasculinization of male frogs (Hayes *et al.* 2002). This hypothesis was based on experiments with adult male *X. laevis* that showed reduced plasma T concentrations after treatment with atrazine (Hayes *et al.* 2002). However, a different study with adult male *X. laevis* exposed to atrazine concentrations ranging from 1 to 250 μg/L showed no significant effects on plasma T concentrations or aromatase activity (Hecker *et al.* 2005), or on *CYP-19* mRNA expression (Park *et al.* 2005). A field study conducted in South Africa, where *X. laevis* is native, found negative correlations between T concentrations and environmental atrazine concentrations, but no effects on aromatase activity were observed (Hecker *et al.* 2004).

Studies in human cell systems found that atrazine increased aromatase activity at concentrations between 0.3 and 30 μM (64.71 and 6471 μg atrazine/L) (Sanderson *et al.* 2000, 2001). However, a series of studies conducted to confirm this mode of action did not find any significant effects of atrazine on aromatase *in vitro* or *in vivo* (Crain *et al.* 1997, Heneweer *et al.* 2003, Hecker *et al.* 2004 and 2005, Kazeto *et al.* 2004, Coady *et al.* 2005). A recent study using *in vitro* binding assays found that 5 nM atrazine (1.08 μg atrazine/L) significantly inhibited phosphodiesterase activity; the authors hypothesized that phosphodiesterase inhibition could result in elevated concentrations of the secondary messenger cAMP, leading to an up-regulation of aromatase activity (Roberge *et al.* 2004).

Plasma 11-ketotestosterone (KT) concentrations have not been reported previously in frogs, but KT is known to be an important androgen in males of many teleost fish species, where it is synthesized from 11β-hydroxytestosterone (Kime 1993). Given the close phylogenetic relationship between fish and amphibians, it seemed possible that KT could be measured in frog plasma and that it might also have a role in androgen-mediated cycles in male frogs. This hormone was therefore included in this study in the context of determining potential atrazine effects in frogs.

The goal of this study was to determine whether hypothesized atrazine-induced effects on steroidogenesis were occurring in native ranid frogs by measuring estradiol (E2), T and 11-ketotestosterone (KT) concentrations in blood plasma and aromatase activities in the gonads of the green frog (*Rana clamitans*), bullfrog (*R. catesbeiana*) and Northern leopard frog (*R. pipiens*) collected from agricultural and non-agricultural sites in south central and south western Michigan.

Materials and methods

Site selection and frog sampling

Site selection and frog collection procedures used in this study can be found in Chapter 1. Briefly, agricultural (corn-growing) and non-agricultural sites were sampled twice each year in the summers of 2002 and 2003. Study sites were selected on the basis of potential atrazine exposure and the presence of relatively large populations of ranid frogs. Sites were located in three regions in south-central Michigan: Kalamazoo, the greater Lansing area (GLA) and Lapeer (LPR) (Figure 1). In the first year of the study, non-agricultural sites were located in the GLA and LPR regions only; in the second year, non-agricultural sites were located in all three regions. Study sites were selected on the basis of atrazine exposure and the presence of relatively large populations of ranid frogs. Detailed site descriptions and atrazine concentrations can be found in Chapter 1 and Appendix A.

The primary species of interest in this study was the green frog (*R. clamitans*), which is the most common pond frog in the lower peninsula of Michigan (Harding 1997). Green frogs are territorial breeders that are associated with the aquatic environment throughout their lives and are faithful to their pond or wetland habitats (Martof 1953, Harding 1997). However, other ranid species including bullfrogs (*R. catesbeiana*) and Northern leopard frogs (*R. pipiens*), were collected as well in order to investigate interspecies differences in the measured biomarkers.

Juvenile and adult frogs were collected once each year in the summers of 2002 and 2003. Adults were sampled in August and September in 2002 and in May in 2003. In 2003, one site was re-sampled for adults in September to allow comparisons to be made to the previous year's data. Juveniles were sampled in July of both years. Frogs were collected at night using hand nets and buckets. The target sample size for each sampling event was between 40 and 50 frogs per site per age class, and the minimum number collected was 17 frogs. Frogs were anesthetized in 250 mg/L MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO, USA), and each frog was weighed and its snout-vent length (SVL) was measured. 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.

Blood and tissue sampling

Blood was taken from each frog using heparinized syringes (10 mg/ml heparin in 0.9% NaCl) via cardiac puncture. Blood was collected from adults only in 2002 and from both juveniles and adults in 2003. All blood was collected within a four-hour time window to reduce the effects of diurnal cycles on hormone levels. A longitundinal cut was made in the abdomen, and the gonads were visually inspected and photographed. If gonads were deemed to have normal morphology, the right gonad was dissected out and flash-frozen in liquid nitrogen for use in the ^{3}H-water-release aromatase assay, while the remaining gonad was left in the body and the entire frog was preserved in Bouin's solution (Sigma) for subsequent histological analysis (Chapter 1). Because histology was deemed to be a more critical endpoint than aromatase activity, gonads that appeared abnormal during gross examinations were left in the body and the entire frog was preserved in Bouin's solution, meaning no gonad was collected for aromatase measurement. Gonads from adult frogs were weighed prior to freezing, while those of juveniles were too small in most cases (<0.001 g in males) to weigh accurately in the field.

A gonadosomatic index (GSI) value was calculated for adult frogs (Equation 1).

$$GSI = (\text{gonad weight/body weight})*100 \qquad (1)$$

Plasma hormone measurements

Blood was centrifuged at 10,000 rpm for 15 min at room temperature to separate the plasma fraction. Plasma was collected and stored at -80° C until extraction. Plasma samples were extracted twice in ethyl ether, evaporated to dryness under nitrogen and reconstituted in Phosgel buffer. Testosterone (T), estradiol (E2) and 11-ketotestosterone (KT) concentrations were measured using competitive enzyme-linked immunosorbent assays (ELISA's) developed by Cuisset *et al.* (1994) with modifications described by Hecker *et al.* (2002). In the assay, the plasma steroid hormone of interest competes with acetylcholinesterase-labeled steroid for binding sites on a rabbit polyclonal antisteroid antibody. T and KT antibody was obtained from D.E. Kime (University of Sheffield, Sheffield, UK). The characteristics of these antibodies as well as their cross-reactivities have been reported elsewhere (Nash *et al.* 2000). E2 antibody (Cayman Chemical, Ann Arbor, MI) cross-reacted with estradiol-3-glucuronide (17%), estrone (4%), estriol (0.57%), T (0.1 %) and 5α-dihydrotestosterone (DHT) (0.1%); all other steroids cross-reacted with the E2 antibody at less than 0.1%. Accurate measurement of KT, which has previously not been measured in frogs, was verified by spiking experiments with DHT, which has a similar structure and is known to be an active androgen in fish (Kime 1993). Plasma from two frogs was spiked with 100, 250 and 500 pg/ml DHT, extracted and tested in the ELISA. Cross-reactivity of the KT antibody to DHT was found to be 6.1%. The working range for both T and E2 was 0.78-800 pg/well; the working range for KT was 0.19-400 pg/well. Only T and E2 were measured in juveniles in 2003 due to insufficient quantities of plasma.

Aromatase activity

Aromatase activity was measured using methods developed by Lephart and Simpson (1991) with modifications. Briefly, gonads were homogenized in ice-cold phosphate assay buffer (50 mM KPO_4, 1 mM EDTA, 10 mM glucose-6-phosphate, pH 7.4), and 500 uL of the homogenate was used in the assay. Adult and juvenile gonads were homogenized in 900 and 600 uL assay buffer, respectively. Reagents were added to the homogenate as follows: 54 nM ^{3}H-androstendione (25.9 Ci/nmol; Lot No. 3467-067; cat. no. NET-926; New England Nuclear, MA, USA), 10 mM NADP (Sigma) and 100 IU glucose-6-phosphate dehydrogenase (Sigma). The reaction was allowed to proceed at 37°C for 120 min. The tritiated-water end product of the reaction was extracted using chloroform and measured on a LS6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA). Aromatase activity was expressed as fmol androstendione converted per h per mg protein. The specificity of the reaction for the substrate was determined by use of a competitive test with non-labeled androstenedione. Addition of 7.5 µl of 5.6×10^2 µM 4-androsten-4-ol-3,17-dione reduced tritiated water formation to the levels found in the tissue blanks, which demonstrated that the activity being measured was specific for aromatase. Protein concentrations were determined using the Bradford

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assay with bovine serum albumin (BSA) (Sigma) as a protein standard (Bradford 1976). The lowest detectable activity for the tritium-release assay was 0.58 fmol/hr/mg prot.

Statistical methods

Data were tested for normality using the Kolmogorov-Smirnov test with Lilliefors transformation and probability plots. GSI, aromatase activities and hormone concentrations were normalized if necessary using ln transformation. If the ln-transformation did not result in a normal distribution, comparisons between sites and between agricultural and non-agricultural sites were determined using nonparametric Kruskal-Wallis tests. Pearson pair-wise (product moment) correlations were used to test for relationships between atrazine concentrations and median GSI values and hormone concentrations. Pearson correlations were used to test for relationships between aromatase activity and atrazine concentrations in females, while Spearman (non-parametric) correlations were used to test for these relationships in males where these parameters were not normally distributed. Power analyses were conducted to determine the ability to detect significant differences within the framework of the study. All analyses were conducted using Systat 11 (SSI, Richmond, CA, USA). Significance level was set at $\alpha<0.05$ for all statistical tests.

Results

All of the measured parameters exhibited significant variability within and among sites (Figures 5-10). Due to the larger sample size obtained for green frogs, statistical analyses were conducted primarily on this species. However, to attempt to determine interspecies differences in the measured parameters, comparisons with bullfrogs and leopard frogs were made whenever possible.

GSI and hormone concentrations

Males

With the exception of GSIs, all parameters measured showed considerable variation both among and within sites during both sampling seasons (Figures 5 & 6). While GSIs were similar in male frogs collected in both years, there was a general trend to greater plasma androgen levels, and lower plasma E2 concentrations in early summer 2003 when compared to late summer 2002.

In 2002 GSI, E2, T and KT concentrations, as well as the ratios, E2/T and KT/T of adult male green frogs were significantly different among all of the locations (Figure 5, Table 6). Median E2 and T concentrations in male green frogs were greatest at site NA3 (T=18.3 ng/ml, E2=17.5 ng/ml), while median KT was greatest at site NA1 (0.19 ng/ml). GSI, E2/T and KT/T differed significantly between agricultural and non-agricultural sites, with significantly greater values observed at the agricultural sites (Table 6). However, power analysis indicated that the power to detect a 4-fold difference in T or E2 concentrations, which was deemed biologically relevant, was below 0.20. Hormones were not measured in juvenile plasma in 2002.

In 2003, all measured parameters except GSI were significantly different among sites in adult males (Figure 6, Table 6). Median E2 concentration was greatest at site NA4 (0.74 ng/ml) and median T was greatest at site Ag8 (27.4 ng/ml) in adult male green frogs. KT concentrations were greater overall in 2003 compared to 2002, with the greatest median concentration at site Ag8 (0.68 ng/ml). Concentrations of all three hormones were less in the adult male frogs sampled in the fall at site Ag2 compared with those sampled at this site in early summer. T, E2/T and KT/T differed significantly between agricultural and non-agricultural sites. T concentrations and KT/T values were significantly greater at agricultural sites, while E2/T values were higher at non-agricultural sites (Table 6). Power analysis indicated that a 1.5-fold difference in GSI could be detected (β=0.933), but the ability to detect 4-fold differences in E2 was 0.37. Concentrations of T and E2 and the ratio E2/T were significantly different among sites in juvenile male green frogs in 2003, but only T concentration and E2/T ratio were significantly different between agricultural and non-agricultural sites (Figure 9, Table 6). The power to detect differences in E2 concentrations was found to be less than 0.10. The greatest median T and E2 concentrations were measured in juvenile green frogs from site Ag9 (T=1.42 ng/ml, E2=0.28 ng/ml), but the greatest and most variable concentrations overall were measured in juvenile green frogs from site NA3. T concentrations were generally less in plasma of juvenile green frogs than in adults, while E2 concentrations were comparable to and in some cases greater than those measured in adults. KT concentrations were generally greater in frogs collected in 2003.

GSI was less in adult male bullfrogs than in adult male green frogs at all sites where both species were collected in both years of the study. Plasma concentrations of T and KT in adult male leopard frogs in 2002 were comparable to or greater than those measured in adult male green frogs and bullfrogs, but concentrations in adult male leopard frogs collected in 2003 were in the lesser range or less than those measured in the adult male green or bullfrogs.

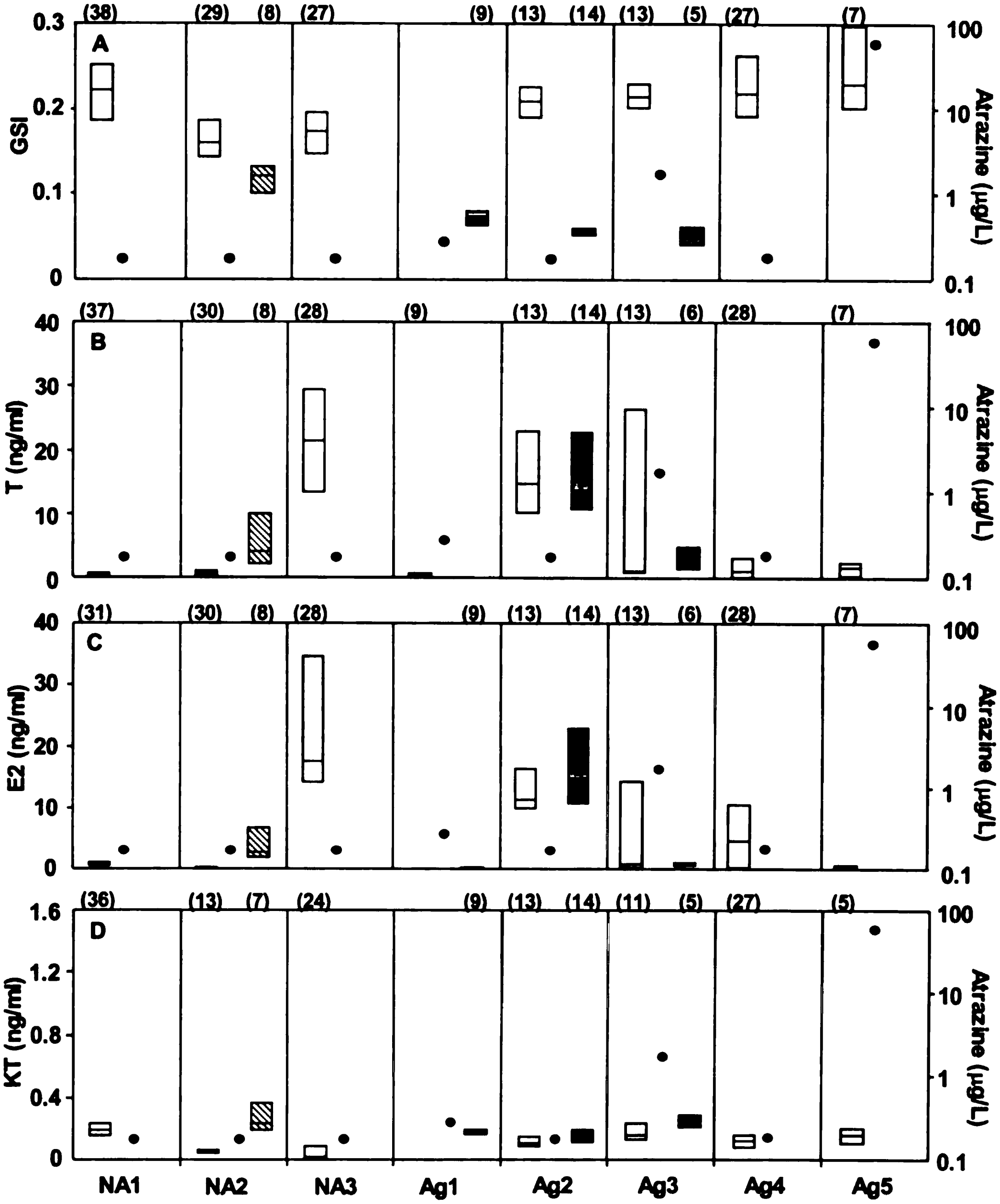

Figure 5. GSI (A) and T (B), E2 (C), and KT (D) concentrations measured in adult male frogs in 2002. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. Open bars represent *R. clamitans*, shaded bars represent *R. catesbeiana* and cross-hatched bars represent *R. pipiens*. The numbers in parentheses above the graphs represent sample size for each species. Points represent the 75% quartile of the atrazine concentrations measured in water from the sites.

Table 6. Inter-site and land use comparisons using Kruskal-Wallis and Mann-Whitney U tests.

[a] Statistical tests could not be conducted on aromatase activities in adult or juvenile males in either year of the study because 80-90% of activities in males were less than the detection limit of the assay.

	Sex	Year	Age Class	GSI	T	E2	E2/T	11KT	11KT/T	Aromatase[a]
Site Differences	Males	2002	Adults	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
			Juveniles							
		2003	Adults	0.200	<0.001	<0.001	<0.001	<0.001	<0.001	
			Juveniles		<0.001	0.002	<0.001			
	Females	2002	Adults	0.001	<0.001	<0.001	<0.001	0.001	<0.001	0.002
			Juveniles							<0.001
		2003	Adults	0.311	0.146	0.045	0.112	0.005	0.027	0.769
			Juveniles		0.005	<0.001	<0.001			0.137
Non-Agricultural vs. Agricultural	Males	2002	Adults	<0.001	0.906	0.423	0.046	0.226	0.041	
			Juveniles							
		2003	Adults	0.156	0.009	0.055	0.001	0.080	<0.001	
			Juveniles		<0.001	0.188	<0.001			
	Females	2002	Adults	0.075	0.102	0.885	0.011	0.001	<0.001	<0.001
			Juveniles							0.004
		2003	Adults	0.786	0.153	0.436	0.673	0.124	0.753	0.507
			Juveniles		0.004	0.246	0.852			0.044

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Females

Adult females in 2002 differed significantly among sites in terms of GSI, T, E2, E2/T, KT and KT/T (Figure 7, Table 6). The greatest median T and E2 concentrations in adult female green frogs were measured at site NA3 (T=17.2 ng/ml, E2=21.4 ng/ml), while the greatest median KT concentration was measured at site Ag3 (0.19 ng/ml). E2/T, KT and KT/T were significantly different at agricultural sites compared to non-agricultural sites, with greater values at agricultural sites (Table 6). The power to detect differences in GSI, T and E2 in 2002 was less than 0.20.

In 2003, E2, KT and KT/T differed significantly among sites, but none of the measured biomarkers differed between agricultural and non-agricultural sites (Figure 8). The power to detect differences in GSI was determined to be 0.09, while the power for E2 and T was 0.30 and 0.15, respectively. KT concentrations were greatest over all adult frogs of all three species sampled in both years of the study in adult female green frogs collected in 2003. Juvenile female green frogs from 2003 differed significantly among sites in E2 and T concentrations and E2/T (Figure 9, Table 6). Of these parameters, only T concentrations differed between agricultural and non-agricultural sites, with greater concentrations measured at agricultural sites (Table 6). The power to detect differences in E2 concentrations was determined to be 0.10.

All three species were comparable in terms of GSI in both years of the study, and comparable in hormone concentrations in 2002. However, in 2003, hormone concentrations in plasma of adult female bullfrogs and green frogs were similar, while those of leopard frogs were consistently less.

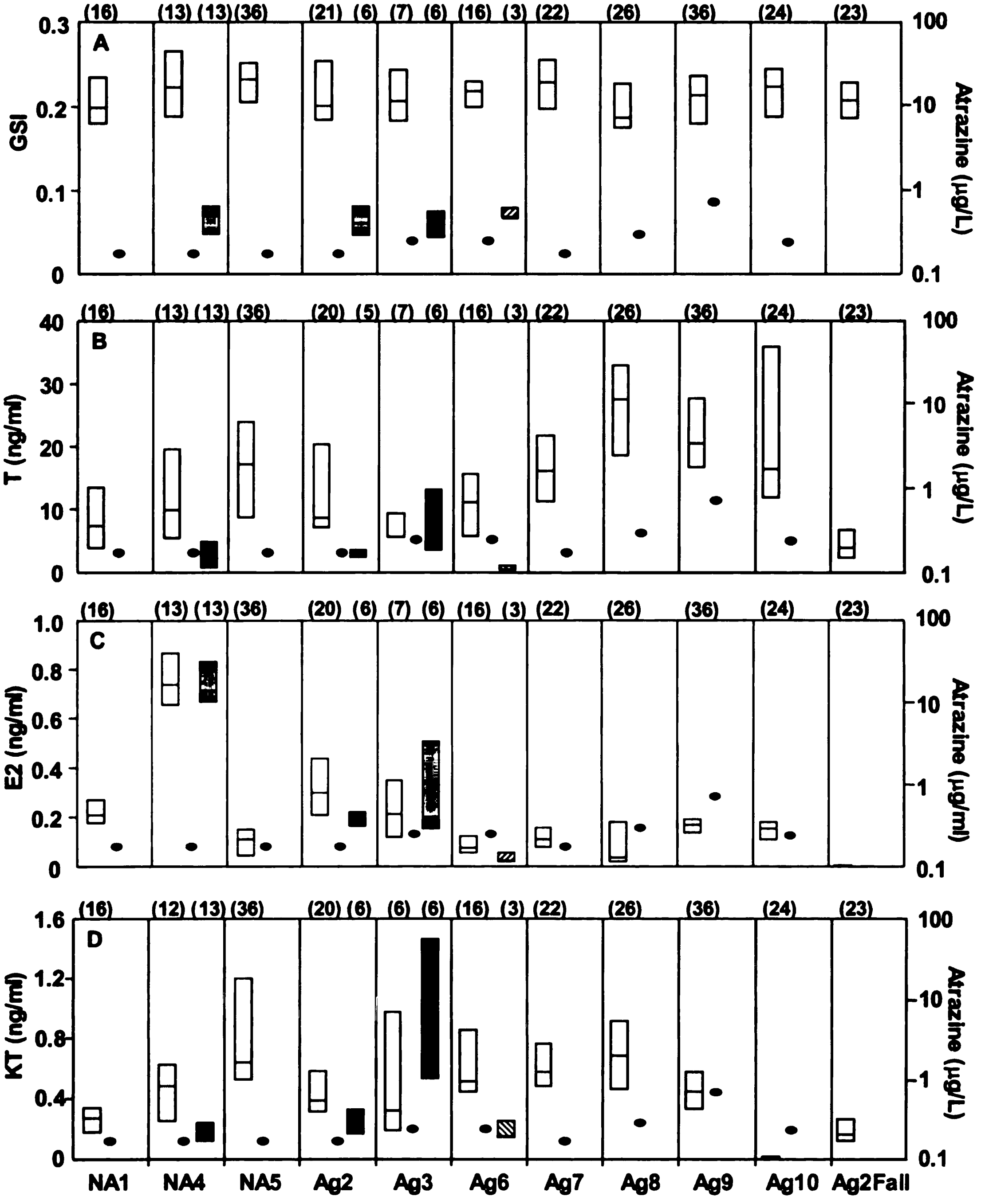

Figure 6. GSI (A) and T (B), E2 (C), and KT (D) concentrations measured in adult male frogs in 2003. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. Open bars represent *R. clamitans*, shaded bars represent *R. catesbeiana* and cross-hatched bars represent *R. pipiens*. The numbers in parentheses above the graphs represent sample size for each species. Points represent the 75% quartile of the atrazine concentrations measured in water from the study sites.

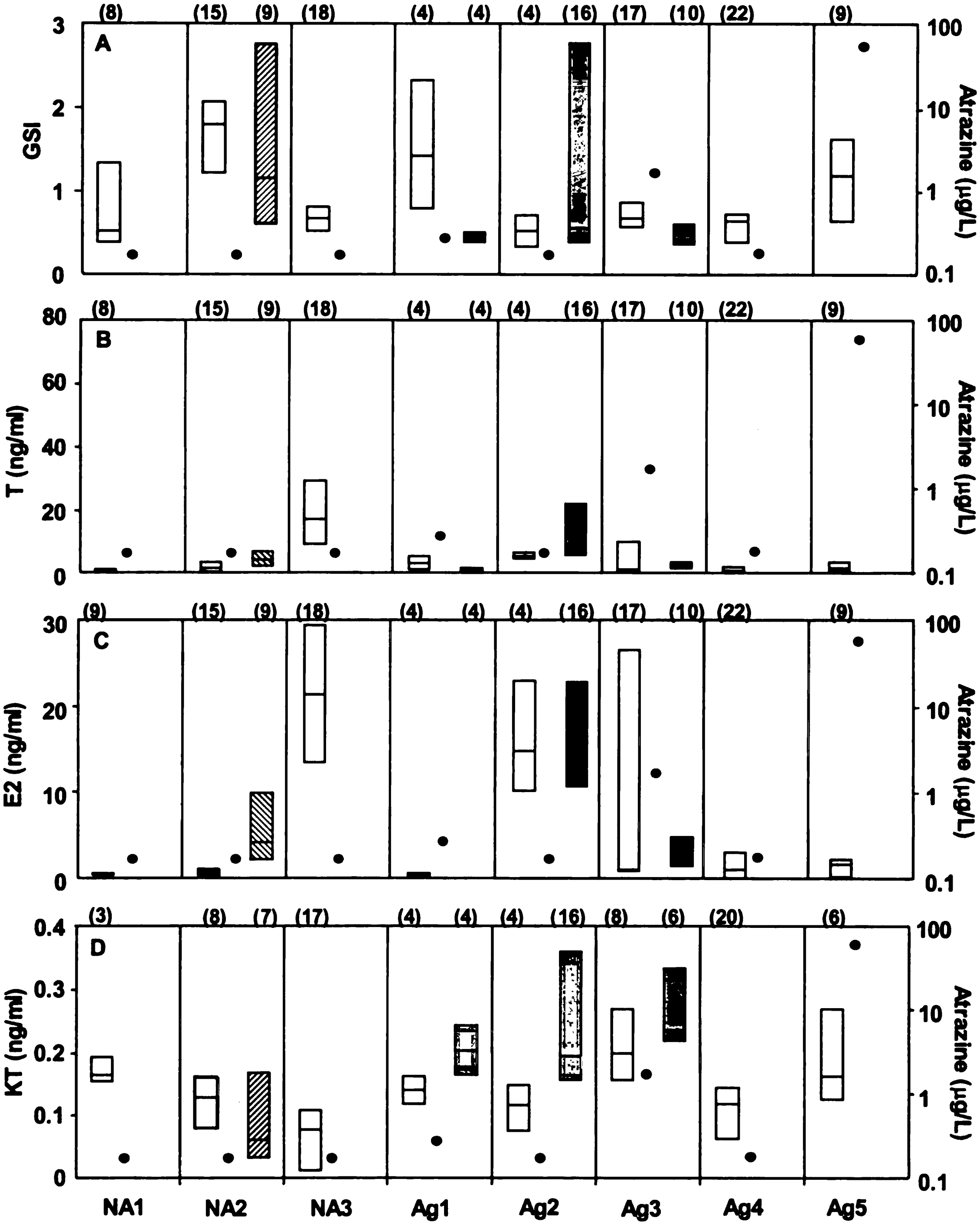

Figure 7. GSI (A) and T (B), E2 (C), and KT (D) concentrations measured in adult female frogs in 2002. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. Open bars represent *R. clamitans*, shaded bars represent *R. catesbeiana* and cross-hatched bars represent *R. pipiens*. The numbers in parentheses above the graphs represent sample size for each species. Points represent the 75% quartile of the atrazine concentrations measured in water from the study sites.

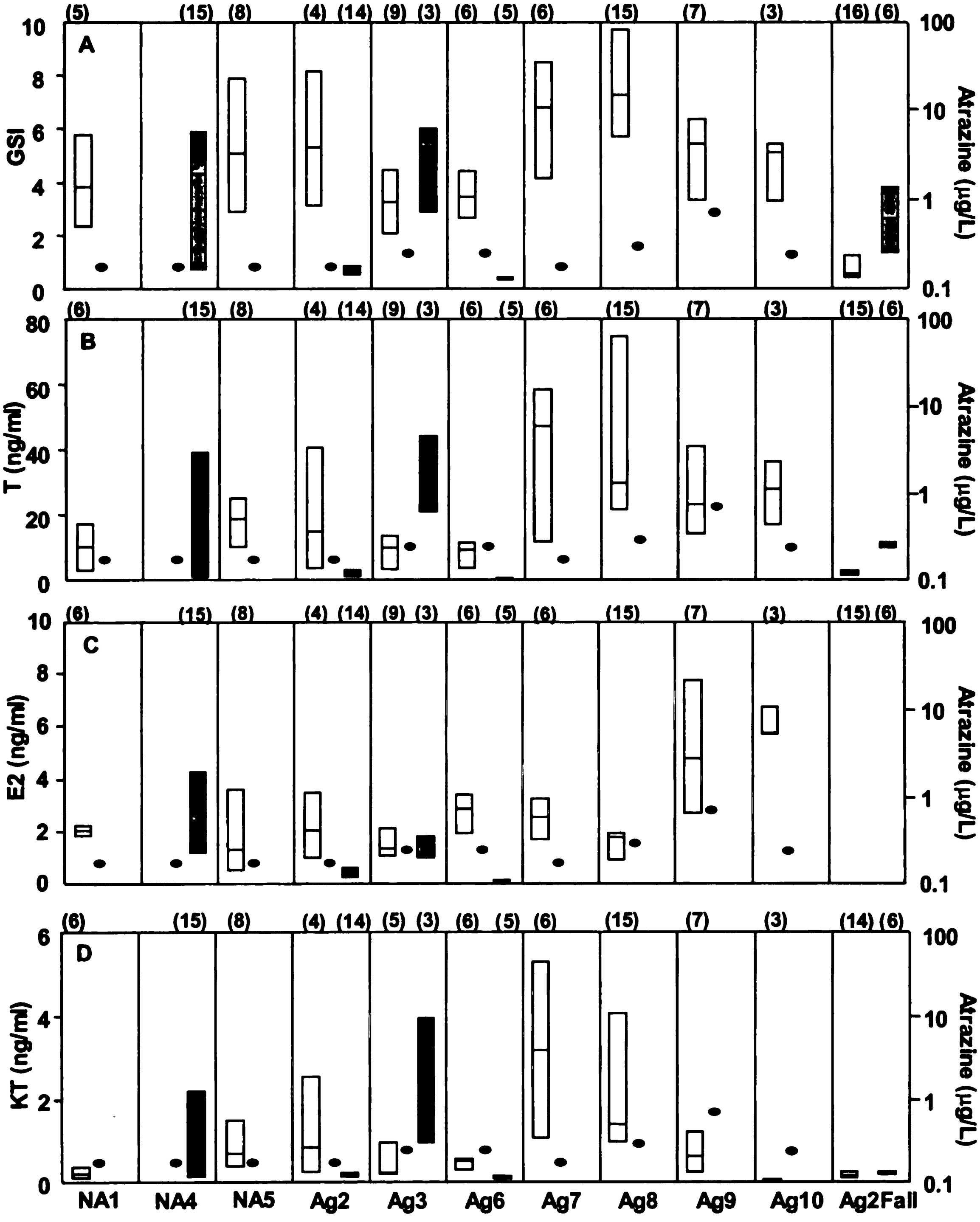

Figure 8. GSI (A) and T (B), E2 (C), and KT (D) concentrations measured in adult female frogs in 2003. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. Open bars represent *R. clamitans*, shaded bars represent *R. catesbeiana* and cross-hatched bars represent *R. pipiens*. The numbers in parentheses above the graphs represent sample size for each species. Points represent the 75% quartile of the atrazine concentrations measured in water from the study sites.

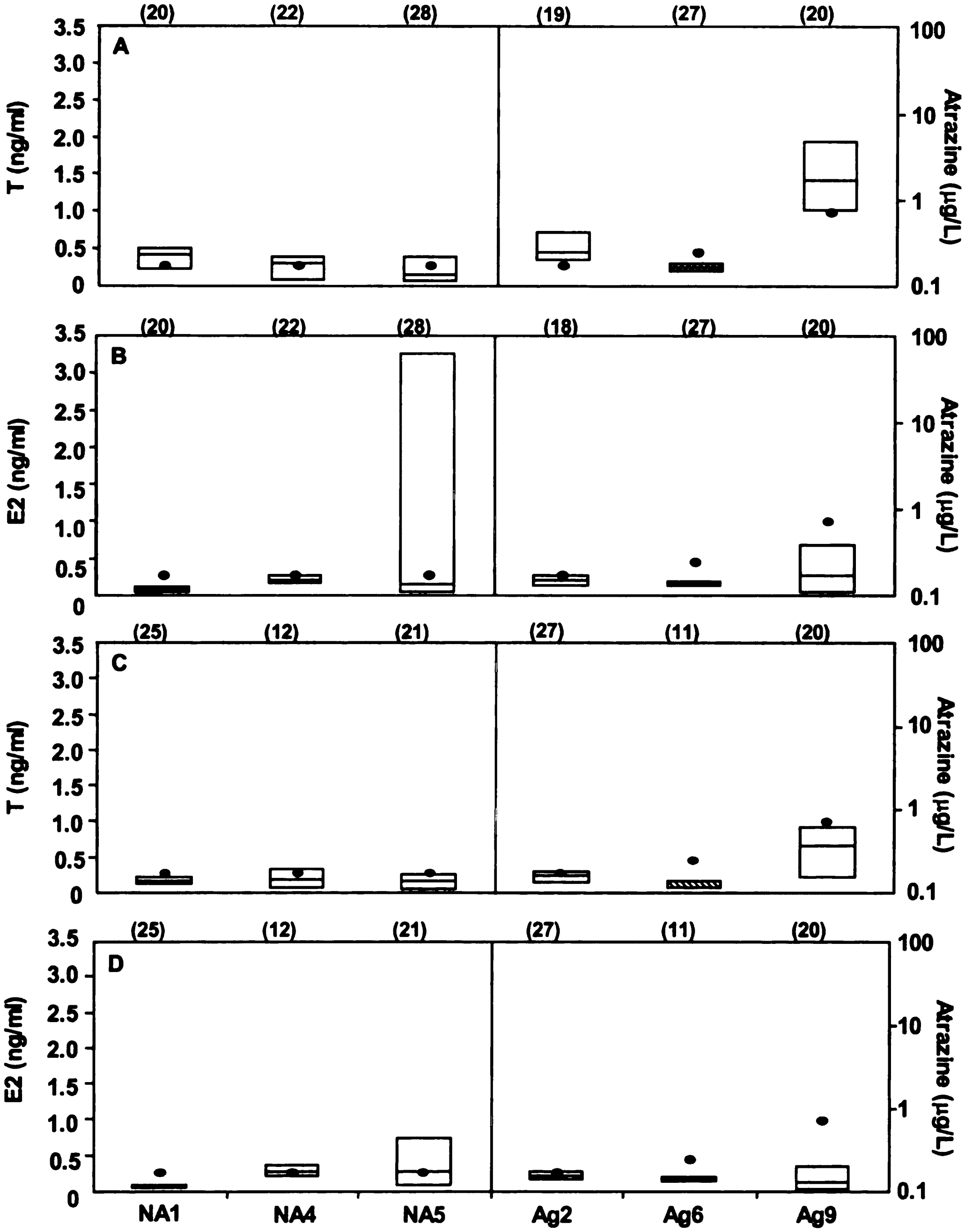

Figure 9. Juvenile male T (A) and E2 (B) and juvenile female T (C) and E2 (D) concentrations in 2003. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. Open bars represent *R. clamitans* and cross-hatched bars represent *R. pipiens*. The numbers in parentheses above the graphs represent sample size for each species. Points represent the 75% quartile of the atrazine concentrations measured in water from the study sites.

Aromatase activities

Males

Aromatase activities were less than the lowest measured activity in the assay (0.58 fmol/hr/mg prot) in most of the testes analyzed. Non-detectable values were set to one-half of the least measured value over all samples analyzed. These values were 0.29 and 0.04 fmol/hr/mg protein for adults and juveniles, respectively.

Due to the low number of detectable activities in both adult and juvenile male frogs, statistical tests could not be conducted on these values. Only 19 adult males (10.4%) in 2002 had activities greater than the detection limit of the assay; of these, only 3 had activities greater than 20 fmol/h/mg prot. The maximum measured activities were found in two frogs; one adult green frog was found to have an aromatase activity of 200 fmol/h/mg prot, while the activity in one adult bullfrog was 440 fmol/h/mg prot. Both of these frogs were from site Ag2. In juvenile green frogs, only 6 of the 169 males analyzed (3.6%) had activities greater than the detection limit of the assay, and of these the greatest activity measured was 1.63 fmol/h/mg prot.

Aromatase activities in adult male green frogs in 2003 were above the assay detection limit in 28 of 249 males (11.2%); of these frogs, all but one had activities that were less than 7 fmol/h/mg prot. One adult male green frog at site NA5 had an activity of 187 fmol/h/mg prot. Aromatase activities of all juvenile green frogs were less than the detection limit with the exception of one green frog from site Ag2 which had an activity of 71 fmol/h/mg prot.

Females

Aromatase activities in ovaries of female green frogs were much greater than those in the testes of males. However, aromatase activity was still less than the detection limit of the assay in 7 of 136 adults (5.1%) and 55 of 124 juveniles (44.4%) collected in 2002. Aromatase activities in adult females ranged from less than the assay detection limit to greater than 1700 fmol/h/mg prot (Figure 10). Activities in juvenile female green frogs were comparable to or in some cases greater than activities in adults. In 2002 aromatase activities in ovaries of adult female green frogs were significantly different among locations and the activities of adult, female, green frogs were greater in frogs from agricultural areas (Table 6). Aromatase activities in the ovaries of juvenile female green frogs were also significantly different among sites, with greater activities at agricultural sites.

Aromatase activity in females in 2003 was less than the assay detection limit in 15 of 101 adults (14.9%) and in 17 of 150 juveniles (11.3%) collected. Aromatase activities in the ovaries of adult female green frogs were not significantly different either among sites or between agricultural and non-agricultural sites (Figure 10, Table 6). Aromatase activity in the ovaries of juvenile female green frogs was not significantly different among sites, but was significantly greater in the agricultural regions than the non-agricultural regions.

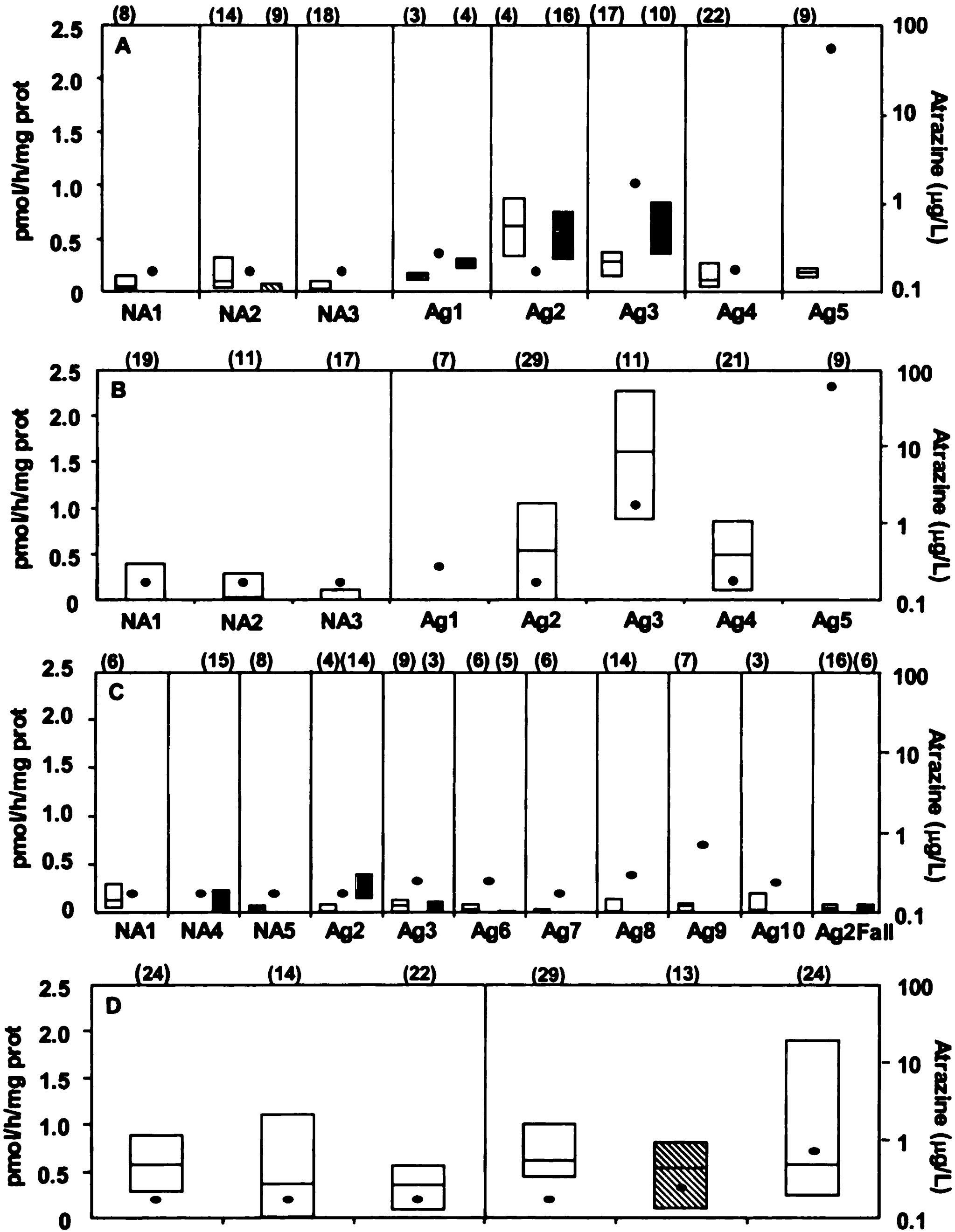

Figure 10. Aromatase activities measured in adult (A) and juvenile (B) female frogs in 2002 and in adult (C) and juvenile (D) females in 2003. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. Open bars represent *R. clamitans*, shaded bars represent *R. catesbeiana* and cross-hatched bars represent *R. pipiens*. The numbers in parentheses above the graphs represent sample size for each species. Points represent the 75% quartile of the atrazine concentrations measured in water from the study sites.

Atrazine correlations

Correlations were determined between atrazine concentrations measured approximately 4 wk before collection of the frogs at a site (4WATZ) and those measured at the time of sampling (ATZ) and the hormone concentrations, their ratios and aromatase activities. Atrazine concentrations were not correlated with GSI, T, E2, E2/T, KT, KT/T or aromatase activity in adult or juvenile green frogs collected in either 2002 or 2003.

Discussion

Seasonal patterns

The variability observed in plasma hormone concentrations can largely be explained by seasonal factors. Adults collected in early summer 2003 before spawning had greater hormone concentrations than those collected in 2002, while those collected at the end of the summer in 2002 were more variable. Studies of seasonal hormone profiles in frogs have found that concentrations decline rapidly once the breeding period begins (Licht *et al.* 1983, Fasano *et al.* 1989, Ko *et al.* 1998). However, hormone concentrations in species with longer breeding periods, such as the green frog and bullfrog, may peak more than once per breeding season to allow for the possibility of repeat spawning (Licht *et al.* 1983). Whether or not a frog breeds more than once is likely due to a combination of climatic and resource-based factors, such as late frosts and prey availability. Changes in these environmental conditions may favor early breeders over late breeders in one year and then favor the opposite the following year, factors which contribute to the kind of variability in reproductive condition observed in this study.

Different breeding strategies also affect seasonal GSI patterns. GSI in males of all three species collected was not as variable over the summer as the other parameters measured. This finding is consistent with previous research that has shown that different species have different breeding strategies. For example, a study on three species of frogs in Korea that breed in the spring and early summer found that GSI in *R. rugosa* did not vary at all through the year, while *R. nigromaculata* exhibited a slight peak in GSI during the spawning season, and *R. dybowskii* had a large peak and decrease in GSI (Ko *et al.* 1998). GSI in *R. nigromaculata* and *R. dybowskii* peaked in the months following breeding. The variation in GSI was thought to be related to habitat; *R. dybowskii* is found in mountainous habitat where resources are likely to be limited, meaning that energy for sperm production and other reproductive processes must be accumulated and expended during the summer months when resources are available. Like the bullfrog, *R. rugosa* has a relatively long breeding season, but GSI in male bullfrogs was found to fluctuate throughout the breeding season, which is consistent with a strategy where breeding can occur more than once (Licht *et al.* 1983); the sampling scheme in the current study did not capture these fluctuations.

The effects of season on sampling are evident in the wide range of E2 concentrations measured in juvenile males at site NA4 in 2003. This site was sampled twice, with a week between sampling times, and those frogs collected during the second sampling period had much greater concentrations of E2. The reason for this increase in plasma hormone concentration is unknown, but it may be indicative of short term changes in endocrine function in response to an environmental stressor or other factors, including possible environmental triggers of certain reproductive processes. Further research into the seasonal hormone profiles in adult green frogs, as well as the potential effects of environmental factors on these hormones is necessary for a better understanding of these patterns.

Comparisons with leopard frogs are more difficult to make because their breeding period is much shorter than that of the other species. Nevertheless, leopard frogs appeared to have greater concentrations of T, E2 and KT in late summer 2002 than in early summer 2003. These greater concentrations may indicate that leopard frogs begin to synthesize hormones for the following year's breeding season before they enter their winter hibernation. This strategy is plausible because leopard frogs are spring breeders that must spawn shortly after emerging from hibernation. A study on *R. esculenta*, which also has a relatively short breeding period and breeds only once, showed that GSI in females peaked at the time of spawning, then dropped sharply and remained low until the recrudescence period, when it began to climb as the frog entered hibernation (Mosconi *et al.* 1994, Polzonetti-Magni *et al.* 1998). Another study found that E2 and T concentrations in male *R. esculenta* peaked in March prior to spawning and declined sharply once spawning began, then showed a second smaller peak in November during the winter stasis period (Fasano *et al.* 1989). Similarly, the variability seen in green frogs in late summer 2002 may represent the convergence of a long breeding period and relatively late initiation of breeding, factors which do not require this species to begin preparing for the next season before entering hibernation. Research on bullfrogs in California, USA found that females begin building up ovarian tissue in February, while GSI peaked in May when egg laying was at its peak, and then declined until August, when the cycle started again (Licht *et al.* 1983). This study also found a high degree of asynchrony and variability similar to that seen in the current study. At this point, however, the pattern of gonad growth and reabsorption in green frogs is unknown. Local ecological and environmental conditions may also cause different populations of the same species to differ in their development patterns.

The differences among juvenile and adult female green frogs in terms of aromatase activity represent an interesting finding. The activities measured in juveniles would appear to be consistent with ongoing steroidogenesis, but it has been reported that female green frogs may require 2-3 y to reach sexual maturity (Martof 1956). The onset of steroidogenesis has been reported in frog species that metamorphose more rapidly than green frogs, such as *X. laevis* (Kang *et al.* 1995) and *R. curtipes* (Gramapurohit *et al.* 2000), and the aromatase activities measured in this study were comparable to those measured in juvenile *X. laevis* (Coady *et al.* 2005). However, the point at which steroidogenesis begins in green frogs and the roles hormones and enzymes such as aromatase may play outside of sexual maturation are unknown. The elevated aromatase activities that were observed in juvenile females in this study may indicate that the enzyme is playing a role in other maturation and development processes, but more research is needed to determine if this is the case.

Juvenile frogs were sampled in July in both years of the study, but differences in aromatase activities between agricultural and non-agricultural sites were greater in 2002 than in 2003, indicating that climatic and environmental factors may have had an effect. The summer of 2002 was one of the hottest on record in Michigan (National Weather Service 2002), which may have had an effect on time to metamorphosis or growth rate in juvenile frogs. It is also possible that some seasonal cycling of aromatase activity occurs in juvenile females even though they are sexually immature, but this question has not been investigated.

Plasma concentrations of KT have not previously been reported in frogs, although it is known to be a key androgen in fish (Kime 1993). KT concentrations were 10-1000 fold less than T concentrations in both males and females, but appeared to show some seasonality, with greater concentrations measured in June compared to September. Males and females had similar concentration ranges of KT in late summer 2002, but KT concentrations in 2003 were twice as great in females as in males. However, given the relatively low concentrations that were measured at some sites, it is possible that some of the measured concentrations of KT may be accounted for by DHT, which is known to be an active androgen in amphibians in general (Wallace *et al.* 1999) and in green frogs in particular (Coady *et al.* 2004). DHT and T are known to be highly correlated in bullfrogs, with both hormones peaking in spring or early summer, and greater DHT concentrations were measured in females than males (Licht *et al.* 1983). KT measured in the current study showed the same pattern, peaking in the spring at greater concentrations in females. However, similar patterns in concentration between T and KT would be expected if DHT accounted for some of the KT concentrations measured in this study, but similar T and KT patterns were not observed consistently across sites. The best way to determine whether KT was measured accurately would be to confirm these measurements with LC-MS or another analytical method, but unfortunately this confirmation is lacking. Given that this is the first instance where KT has been reported in frogs, the role of this androgen in sexual development and reproduction is unknown.

Atrazine effects

Atrazine concentrations were not correlated with any of the parameters measured in this study. It is therefore unlikely that plasma sex steroid homeostasis in green frogs collected from agricultural field sites in Michigan during this study was affected in the same manner reported by Hayes *et al.* (2002), who found a decrease in plasma T concentrations in frogs exposed to atrazine.

The mechanism of action for atrazine proposed by Hayes *et al.* (2002) states that up-regulation of aromatase by atrazine results in feminization in male frogs. In this study, no effect on aromatase was found in either juvenile or adult frogs collected from atrazine-exposed ponds. Aromatase activity was not measurable in most of the males frogs analyzed. Three adult and one juvenile male frog had activities that could be characterized as typical of a female frog. One explanation for these relatively high activities could be the presence of testicular oocytes (TO) in the testis, which produce estrogens, as hypothesized by Hayes *et al.* (2002). However, in another aspect of this study, the gonads that were preserved in the frogs were analyzed histologically for the presence of TO, and none were found in the other testes of these two frogs (Chapter 1). A comparison of hormone parameters and number of TO indicated no correlation between these endpoints for all parameters (Spearman R<0.100). This lack of correlation, as well as the fact that there were no TO in the testes examined from these individuals, indicates that these relatively great aromatase activities do not appear to have an effect on feminization of the testis. However, there is no way to know whether TO were present in the testes analyzed for aromatase. The two male frogs with the greatest activities were collected from the same site, so it is also possible that their elevated activities represent a response to an unknown chemical or environmental stressor.

The assay conditions used to measure aromatase activity were optimized to give maximum activity and minimum variation, but may not have been optimal relative to field conditions because enzymes in frogs typically function at temperatures of 25-30 °C, rather than the 37 °C that was used in the assay. However, a study of oviductal aromatase in *R. pipiens* found that maximum enzyme activity occurred at 37 °C (Kobayashi *et al.* 1996). In addition, activities were measurable in the majority of females collected, while the majority of male frogs collected showed extremely little activity. This indicates that aromatase in males was not being elevated to female-like levels, and makes it unlikely that minor changes in aromatase enzyme activities that may have been not detectable with the assay would be of any biological relevance. In addition, other studies have found no effect of atrazine on aromatase activity in *X. laevis* juveniles (Coady *et al.* 2005) or adults (Hecker *et al.* 2004 and 2005), as well as no effect of atrazine on *CYP19* gene expression and aromatase activity (Hecker *et al.* in press).

Aromatase activity in juvenile females in 2003 was significantly higher in agricultural sites than non-agricultural sites, but atrazine concentrations were not correlated with aromatase activity in male or female adult or juvenile frogs. It is therefore unlikely that atrazine was responsible for the observed differences, but the possibility that another chemical or mixture of chemicals may have had an effect on aromatase activity cannot be excluded. These results, coupled with those of other recent studies, indicate that the proposed aromatase-mediated mechanism of action for atrazine is unlikely to be occurring in wild ranid frog species from agricultural areas in Michigan.

Further evidence against the aromatase-up-regulation hypothesis is provided by the fact that none of the hormones measured showed a clear relationship with atrazine concentrations. The results of this study are different from those obtained in another field study on *X. laevis*, where atrazine was found to be negatively correlated with T (Hecker *et al.* 2004). The power to detect differences in T concentrations between agricultural and non-agricultural sites in adult male green frogs in the current study was at or below 20%, which means that significant differences in T concentrations may not have been captured. However, T concentrations did not vary more than 2-fold at the beginning of the breeding season in 2003 between agricultural and non-agricultural sites, a difference that does not appear biologically relevant in the context of the variability that was observed across sites.

Significant differences between agricultural and non-agricultural sites were observed in many of biomarkers measured in this study. These differences may be due to exposure to other unknown contaminants or to environmental factors that were not measured. Water was sampled from study sites in 2002 and analyzed for a number of agricultural chemicals, all of which were undetectable (Murphy *et al.* 2005). However, it is impossible to exclude the possibility that other contaminants were present at the sites that could affect hormone concentrations. Naturally occurring compounds, such as humic acids, could also potentially affect hormone concentrations (Steinberg *et al.* 2004). It is also possible that the habitat alteration that accompanies intensive agriculture affects the breeding strategies of frogs resulting in altered hormone production. Although habitat fragmentation and loss is known to have negative effects on frog populations (e.g. Andersen *et al.* 2004), research on the effects of agriculture has produced conflicting results, with some species appearing to be more successful in agricultural habitats than others (Knutson *et al.* 1999, Kolozsvary and Swihart 1999, Gray *et al.* 2004). The interaction of habitat alteration, reproductive behavior and function and reproductive biomarkers in frogs is currently unknown. A recent study found that changes in microhabitat conditions such as degree of shading affected growth rate in wood frogs (*R. sylvatica*) (Skelly 2004). This is only one study on one species, but it is interesting that such relatively small changes in habitat quality can have relatively large effects on growth and development. It is possible that the loss of optimal habitat as a result of agricultural processes might favor those frogs that are able to emerge early from hibernation, claim territories (in the case of males) and breed earlier in the season. However, the lack of consistent differences across the measured parameters may indicate that the observed differences result primarily from natural variability.

In conclusion, the results of this study indicate that the ranid species collected from agricultural ponds were not affected by atrazine in the manner proposed in previous studies (Hayes *et al.* 2002). Aromatase activities were undetectable in the majority of males, and no effects on T concentrations were found that were consistent with the proposed aromatase up-regulation mechanism.

Chapter 3

Sediment TCDD-EQ'S and EROD and MROD Activities in Ranid Frogs from Agricultural and Non-Agricultural Sites in Michigan (USA)

Abstract

In vitro studies have demonstrated atrazine-mediated induction of 7-ethoxyresorufin *O*-deethylase (EROD) activity, an enzyme that is active in metabolism. 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 environmental atrazine concentrations and the liver-somatic index (LSI) and EROD and 7-methoxyresorufin *O*-deethylase (MROD) activities in ranid frogs. 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 were at non-agricultural sites ranged from less than the limit of quantification (0.17 μg atrazine/L) to 0.23 μg atrazine/L, and did not exceed 2 μg atrazine/L at most agricultural sites. Of the measured parameters, only LSI values in adult, male frogs differed significantly between agricultural and non-agricultural 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 13-21 pmol/min/mg protein in adult male green frogs and from 5-13 pmol/min/mg protein in adult female green frogs. Bullfrogs and leopard frogs had greater activities than did green frogs. Atrazine concentrations were negatively correlated with MROD activity in adult male green frogs. LSI and EROD and

MROD activities were not significantly correlated with atrazine concentrations in adult female or juvenile green frogs. These results indicate that atrazine does not appear to have a consistent effect on EROD or MROD activities in wild-caught green frogs.

Keywords: triazine; amphibian; EROD; MROD; metabolism; field study

Introduction

The triazine herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a widely used herbicide in the US and Canada on crops such as corn, sorghum and sugar cane (US EPA 2003). In the Midwestern US, atrazine is used intensively in corn cultivation. 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 of research that 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 posited to occur is via atrazine-mediated up-regulation of aromatase (*CYP19*), the cytochrome p450 enzyme that converts testosterone (T) to estradiol (E2) (Hayes *et al.* 2002). Evidence for this mechanism was provided by several cell 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.* 2005) or expression of *CYP19* mRNA in adult *X. laevis* (Hecker *et al.* 2005).

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 authors 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 authors 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.* 1998). EROD activity is directly associated with the induction of hepatic cytochrome P4501A1. P4501A1 is a mixed-function oxygenase (MFO) enzyme that is part of the cytochrome p450 enzyme superfamily, and whose main function is the metabolism of xenobiotics. The P450 1A 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). P4501A mainly metabolizes polycyclic aromatic hydrocarbons and structurally similar chemicals while 1A2 preferentially oxidizes heterocyclic and aromatic amines. While both enzymes metabolize some substrates, P4501A1 is much more effective at metabolizing 7-ethoxyresorufin (ER) while P4501A2 is more effective at metabolizing 7-methoxyresorufin (MR). Thus, both substrates provide valuable information as to P4501A activity in an organism relative to its exposure to organic compounds.

Induction of EROD is commonly used as a functional measure of exposure of vertebrates to halogenated aromatic hydrocarbons such as dioxins, PCBs and PAH's (Andersson et al. 2000; Kennedy et al. 1996; Smeets et al. 2002). MFO activities in amphibians, though not yet as well-studied as in other vertebrates, have been measured in both laboratory and field studies. Several of these studies indicate that some species of amphibians are capable of the same fold-induction relative to EROD activity as has been observed in rats and and other mammals. However, there is a great deal of variability in response that is associated with species, sex and stage of development (Ertl and Wilson 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 (*R. catesbeiana*) and leopard frog (*R. pipiens*) (Ertl and Wilson 1998). In addition to EROD activity, methoxyresorufin *O*-deethylase (MROD) activity has been used as another hepatic biomarker of exposure in frogs. Although not as highly inducible as EROD, MROD has been found to be more sensitive than other monooxygenases such as benzyloxy-ROD and pentoxy-ROD in amphibians (Huang *et al.* 1998). The aims of this study were to measure EROD and MROD activities as measures of CYP1A1 and CYP1A2 activity in ranid frogs collected from agricultural and non-agricultural areas, and to investigate site differences and correlations with measured sediment TEQ values and water atrazine concentrations.

Materials and methods

Site Selection and Frog Sampling

Site selection, frog sampling procedures and atrazine concentrations can be found in Chapter 1. Briefly, agricultural (corn-growing) and non-agricultural 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 (Figure 1). Non-agricultural 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 and atrazine concentrations can be found in Chapter 1 and Appendix A. The main species of interest in this study was the green frog (*Rana clamitans*), which is the most common pond frog in Michigan (Harding 1997). Green frogs are territorial breeders that are associated with the aquatic environment throughout their lives and are highly faithful to their pond or wetland habitats (Martof 1953, Harding 1997). However, other ranid species including bullfrogs (*R. catesbeiana*) and leopard frogs (*R. pipiens*), were collected as well in order 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 re-sampled for adult frogs in September to 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 per site per age class, and the minimum number collected was 22 frogs. Frogs were anesthetized in MS-222 and euthanized 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 dissected out, weighed and flash-frozen in liquid nitrogen.

The liver-somatic index (LSI) was calculated (Equation 1):

$$LSI = (\text{liver weight}/\text{body weight}) * 100 \qquad (1)$$

Measurement of MFO activities

Liver microsomes were prepared and ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MROD) activities were measured using methods described by Kennedy and Jones (1994) with modifications. The assays were optimized on 96-well plates (Costar) for all species and age classes collected. The working range for both the EROD and MROD assays was 0-120 pmol resorufin/well, and all assays were run using 12 µl of microsome preparations per well. EROD assays were run using 0.3 mM NADPH (Sigma, St. Louis, MO, USA) and 15 µM ethoxyresorufin (ER) per well. MROD assays were run using 0.45 mM NADPH and 7.5 µM methoxyresorufin (MR) per well. ER and MR were obtained from Molecular Probes (Eugene, OR, USA). All assay plates were pre-incubated for 10 min at 30°C prior to NADPH addition, which was determined to be the optimum incubation temperature for EROD and MROD enzyme activities in all species. EROD and MROD assays were incubated at 30°C for 10 and 15 min, respectively, after which the reaction was stopped using 1.08 mM fluorescamine (Sigma). 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 (BSA) (Sigma) as the protein standard (Bradford 1976). Non-detectable 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 chemically dried with sodium sulfate and Sohxlet extracted using dichloromethane (DCM) and hexane (3:1, 400 ml). Extracts were treated with activated copper coils to remove sulfur and evaporated to 11 ml under nitrogen. One ml of extract was removed for use in cell bioassays, and the remaining extract was passed through glass columns containing 10g Florisil for fractionation. The first fraction was eluted with 100 ml hexane, and was expected to contain nonpolar compounds such as PCB's. The second fraction was eluted with 100ml of 20% DCM in hexane, and was expected to contain PAH's, PCDD/F's 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 (DRE's) (Sanderson *et al.* 1996). Cells were cultured at 37°C and 5% CO_2. Cell bioassays were conducted as described elsewhere (Hilscherova *et al.* 2000). Briefly, 250 μl of cell solution per well was seeded into the inner 60 wells of 96-well culture plates (PerkinElmer, Boston, MA, USA). H4IIE-luc cells were seeded at a density of approximately 80,000 cells/ml. Cells were incubated for 24 h, and then dosed with extraction fractions at 1% v/v in triplicate. Solvent controls and blanks were dosed on all plates. Cells were incubated for 72 h and then used in the luciferase assay with 50 μl of Luc-Lite reagent (PerkinElmer) per well. Sample responses were solvent-corrected and compared to dioxin standards ranging from 0.48 to 1500 pM and expressed as pg dioxin equivalents (TEQ).

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 ANOVA. A two-sample *t*-test and Bonferroni adjustment were used to determine differences between agricultural and non-agricultural sites. If data could not be ln-normalized, significant differences between sites and between agricultural and non-agricultural sites were determined using nonparametric Kruskal-Wallis and Mann-Whitney U tests. Pearson correlations were used to test for relationships between atrazine concentrations and median LSI and median EROD and MROD activities. A one-tailed Dunnett's test was used with H4IIE-luc data to determine the first concentration that differed from zero if sample dose-response curves did not meet bioassay assumptions about parallelism and efficacy (Villeneuve *et al.* 2000). All analyses were conducted using Systat 11 (SSI, Richmond, CA, USA). Significance level was set at $\alpha < 0.05$ for all statistical tests.

Results

Due to the larger number of green frogs, statistical analyses were conducted primarily on this species. However, comparisons to 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 are reported in Chapter 1 (Table 2). Briefly, 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 to non-agricultural sites ($p=0.010$, Mann-Whitney U).

AhR activity

Except for one site, sediment TEQ's were less than 1 pg/g dry wt. 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 dose-response curves from which EC50 values could be derived. These values were determined to be 4.64 pg TEQ/g and 4.20 pg TEQ/g dry wt, respectively. None of the other sites had detectable dioxin-like activities.

LSI

LSI values were comparable between adult and juvenile frogs, but overall LSI values were greater in adult female frogs than in male adults or in male or female juveniles (Figures 11-14). The highest median LSI was measured in adult female green frogs at site Ag6 (LSI=3.88). For adult female green frogs, LSI differed significantly among sites (p=0.029, ANOVA), but did not differ significantly between agricultural and non-agricultural sites (p=0.343, *t*-test, Figure 11). For adult male green frogs, LSI also significantly differed among sites (p<0.001, ANOVA), and was significantly greater at agricultural sites than at non-agricultural sites (p=0.001, *t*-test, Figure 12). In juvenile female green frogs, LSI was significantly different among sites (p<0.001, ANOVA), but did not differ between agricultural and non-agricultural sites (p=0.162, *t*-test, Figure 13). LSI in juvenile male green frogs was significantly different among sites (p<0.001, ANOVA), but was not significantly different between agricultural and non-agricultural sites (p=0.079, *t*-test, Figure 14). Power analysis indicated that a 1.25-fold change in LSI was detectable in adult female green frogs at a power ($1-\beta$) of 0.23, and in juvenile green frogs at a power of less than 0.13.

LSI values in adult bullfrogs were comparable to those measured in adult green frogs. However, LSI values were greater in adult female bullfrogs than in adult female green frogs at the site that was re-sampled in the fall (Figure 11). 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 to the other two species.

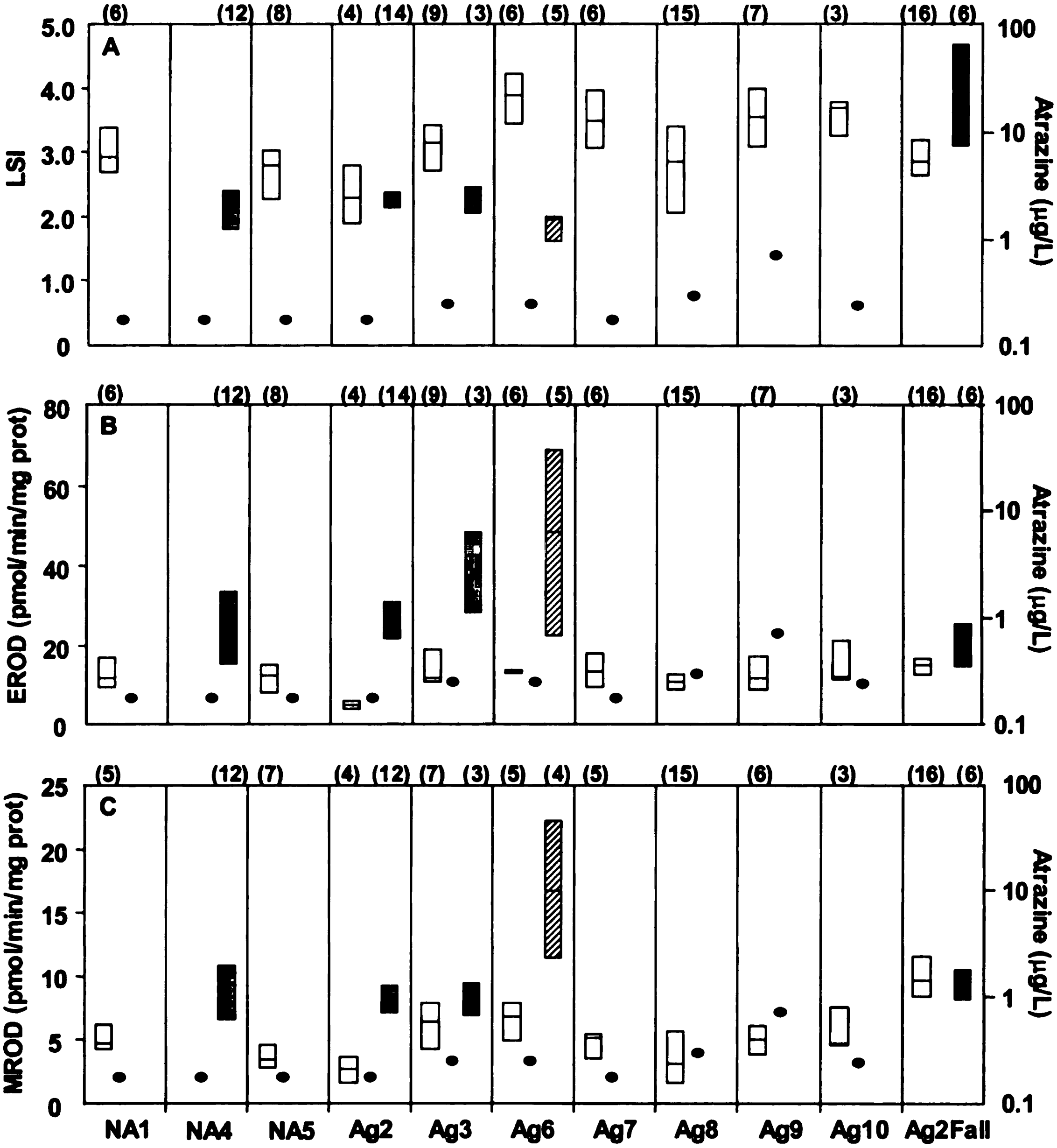

Figure 11. Liver-somatic index (LSI) (A) and ethoxyresorufin *O*-deethylase (EROD) (B) and methoxyresorufin *O*-deethylase (MROD) (C) activities measured in adult female frogs. The horizontal line on each bar represents the median, and bar length represents the middle 50% of the data Open bars represent *R. clamitans*, shaded bars represent *R. catesbeiana* and cross-hatched bars represent *R. pipiens*. The numbers in parentheses above the graphs represent sample size for each species. Points represent the 75% quartile of the atrazine concentrations measured in water from the sites.

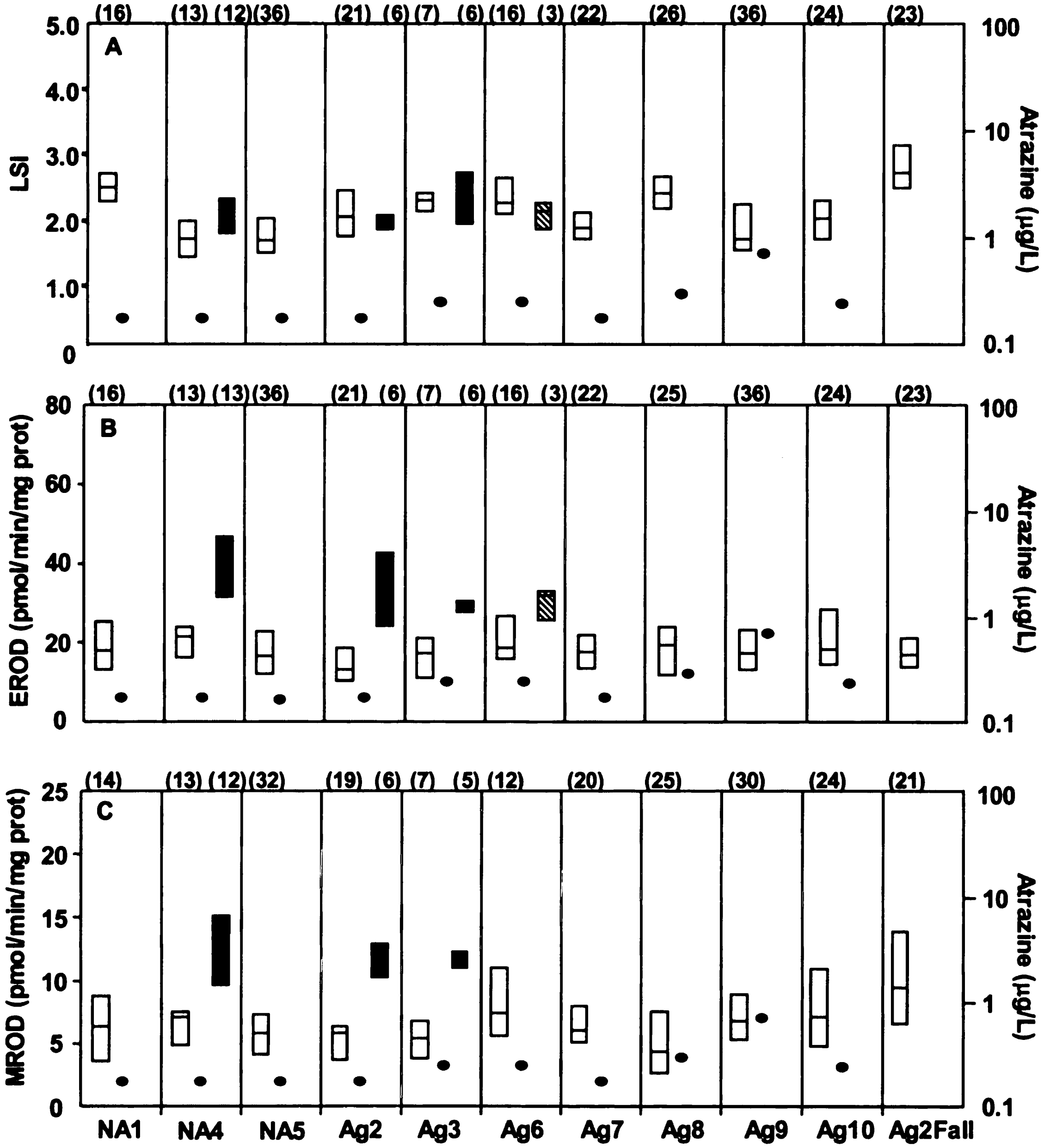

Figure 12. Liver-somatic index (LSI) (A) and ethoxyresorufin *O*-deethylase (EROD) (B) and methoxyresorufin *O*-deethylase (MROD) (C) activities measured in adult male frogs. The horizontal line on each bar represents the median, and bar length represents the middle 50% of the data. Open bars represent *R. clamitans*, shaded bars represent *R. catesbeiana* and cross-hatched bars represent *R. pipiens*. The numbers in parentheses above the graphs represent sample size for each species. Points represent the 75% quartile of the atrazine concentrations measured in water from the sites.

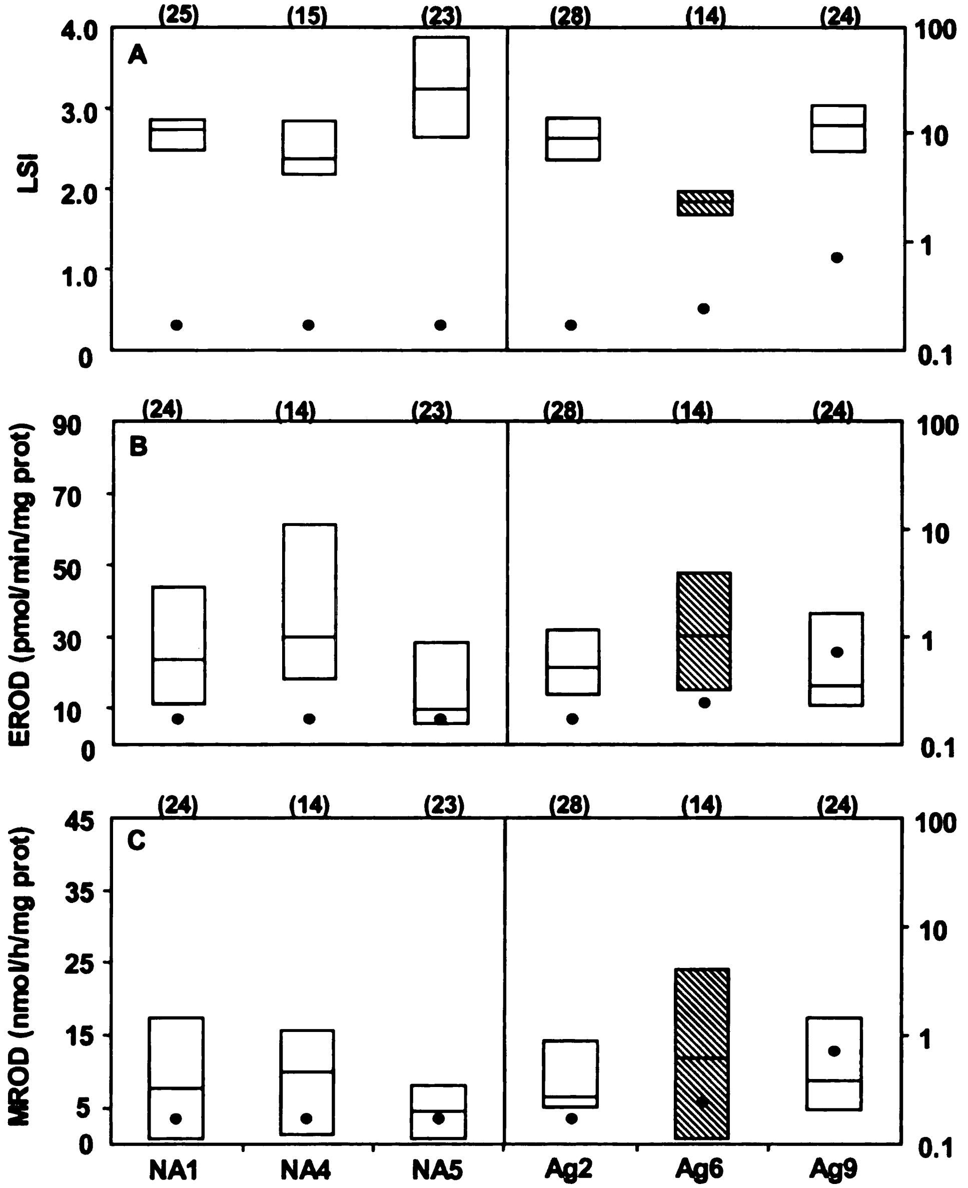

Figure 13. Liver-somatic index (LSI) (A) and ethoxyresorufin *O*-deethylase (EROD) (B) and methoxyresorufin *O*-deethylase (MROD) (C) activities measured in juvenile female frogs. The horizontal line on each bar represents the median, and bar length represents the middle 50% of the data. Open bars represent *R. clamitans*, shaded bars represent *R. catesbeiana* and cross-hatched bars represent *R. pipiens*. The numbers in parentheses above the graphs represent sample size for each species. Points represent the 75% quartile of the atrazine concentrations measured in water from the sites.

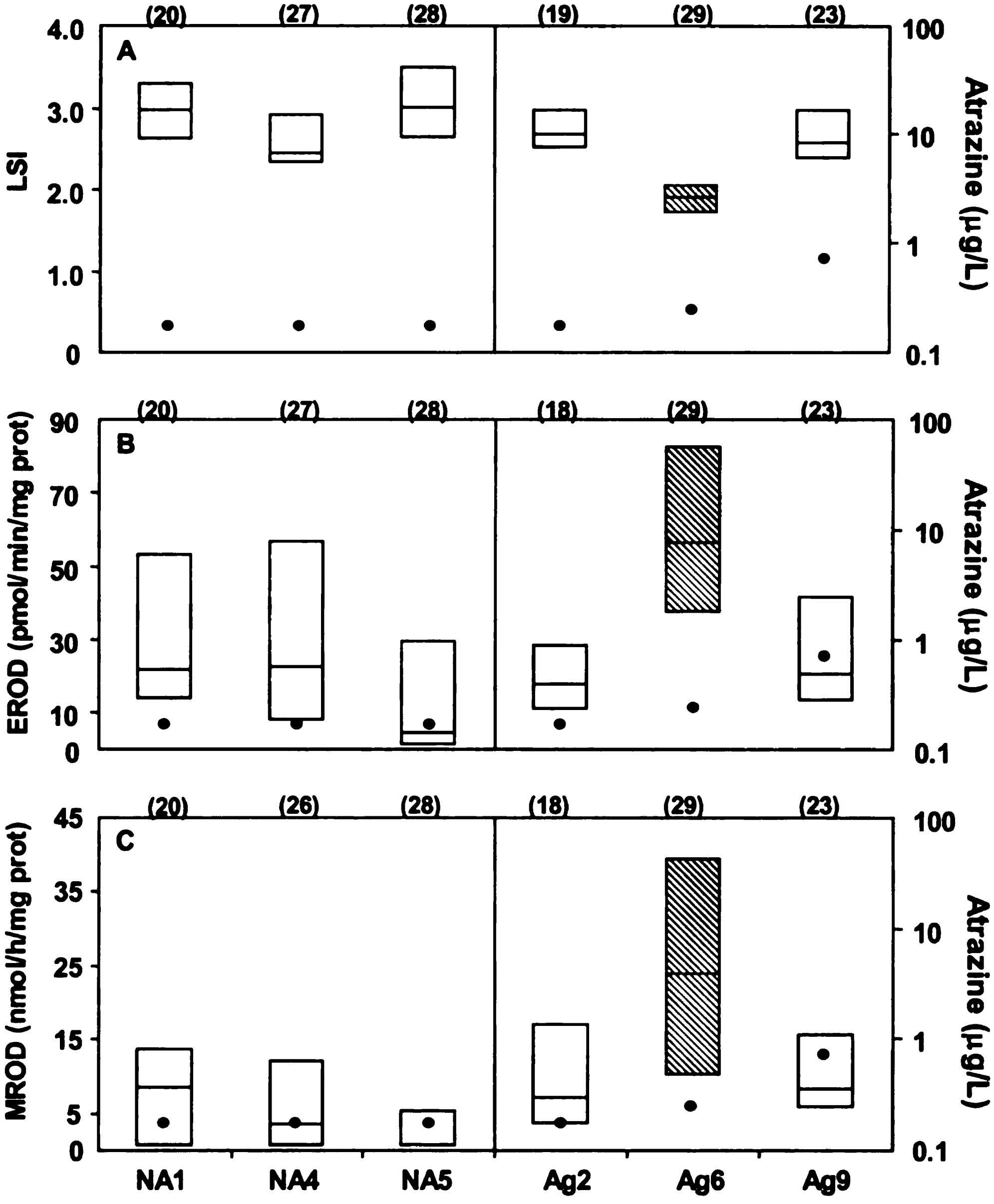

Figure 14. Liver-somatic index (LSI) (A) and ethoxyresorufin *O*-deethylase (EROD) (B) and methoxyresorufin *O*-deethylase (MROD) (C) activities measured in juvenile male frogs. The horizontal line on each bar represents the median, and bar length represents the middle 50% of the data. Open bars represent *R. clamitans*, shaded bars represent *R. catesbeiana* and cross-hatched bars represent *R. pipiens*. The numbers in parentheses above the graphs represent sample size for each species. Points represent the 75% quartile of the atrazine concentrations measured in water from the sites.

EROD and MROD activities

EROD activities were comparable between adult male and female green frogs while EROD activity was greater in juveniles when compared to adults. The greatest median EROD activity in both adult and juvenile green frogs was measured in males at site NA4 (EROD activity=21.4 and 29.8 pmol/min/mg protein, respectively, Figures 12 and 14). For adult female green frogs, EROD activities were significantly different among sites (p=0.044, ANOVA) in adult female green frogs, but did not differ between agricultural and non-agricultural regions (p=0.978, *t*-test, Figure 11). In adult male green frogs, EROD activities did not differ significantly among sites (p=0.418, ANOVA) or between agricultural and non-agricultural regions (p=0.144, *t*-test, Figure 12). EROD activity in juvenile female green frogs also did not differ significantly among sites (p=0.057, Kruskal-Wallis) or between agricultural and non-agricultural regions (p=0.433, Mann-Whitney U, Figure 13). Likewise, EROD activity in juvenile male green frogs was not significantly different among sites (p=0.082, Kruskal-Wallis) or between agricultural and non-agricultural regions (p=0.432, Mann-Whitney U, Figure 14).

In general, MROD activities were typically 2- to-3-fold less than EROD activities, and were greater in juvenile green frogs as compared to adults (Figures 11-14). 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). In adult male green frogs, MROD activities were significantly different among sites (p=0.016, ANOVA), but did not differ between agricultural and non-agricultural regions (p=0.871, ANOVA, Figure 12). In addition, in adult females

MROD activities did not differ significantly among sites ($p=0.056$, ANOVA) or between agricultural and non-agricultural regions ($p=0.738$, *t*-test) in adult females (Figure 11). In juvenile female green frogs, MROD activity was not significantly different among sites ($p=0.213$, Kruskal-Wallis) or between agricultural and non-agricultural regions ($p=0.085$, Mann-Whitney U, Figure 13). In juvenile male green frogs, MROD activity was significantly different among sites ($p<0.001$, Kruskal-Wallis) and was significantly greater at sites in the agricultural region ($p<0.001$, Mann-Whitney U, Figure 14).

The EROD and MROD activities measured in bullfrogs and leopard frogs were greater than those measured in adult and juvenile green frogs (Figures 12 and 13). Furthermore, EROD and MROD activities in juvenile male leopard frogs were as much as 2- and 4-fold greater, respectively, than activities in juvenile male green frogs.

A 2-fold difference in EROD or MROD activity was detectable in adult male green frogs at a power ($1-\beta$) of 0.74 and 0.68, respectively. The power to detect this difference in adult female green frogs was approximately 0.30 for both EROD and MROD activities, and the power to detect this difference 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 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 and the medians of the measured parameters were determined based on concentrations measured both 4 wk prior to sampling (4WATZ) and at the time of sampling (ATZ). Median LSI and EROD and MROD activities were not correlated with 4WATZ or ATZ in adult female green frogs. Median MROD activity was negatively and correlated with 4WATZ 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 TEQ concentrations. LSI and EROD and MROD activities were not significantly different at this site when compared to 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 to the other sites (p<0.017, ANOVA), although sample size at this site was small (n=4). No relationships were observed between TEQ's 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, while MROD activities of adult and juvenile male green frogs were significantly different among sites. The lesser MROD activities measured indicate that MROD may play a more secondary role in metabolism in the investigated species than does EROD. The significantly greater MROD activities at agricultural sites in juvenile male green frogs were most likely due to a relatively high number of non-detectable activities in frogs from the non-agricultural sites. LSI showed the greatest among-site variability of all the parameters measured, and was found to differ significantly among sites in green frogs of both sexes and age classes. In adult female green frogs, the changes in LSI can be explained by metabolic MROD and EROD activities as indicated by the significant and positive correlations. The reason for the lack of correlation between these parameters in males and the negative correlations between EROD and LSI in juveniles 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. Some of the differences in LSI may be due to such metabolic activities that were not captured in this study. The greater LSI in females when compared to males can be explained by the fact that during early summer prior to the spawning season, female green frogs undergo vitellogenesis which takes place in the liver and which was reported to be positively related to liver size in fish (Hecker et al. 2002).

EROD and MROD activities measured in frogs re-sampled in September at site Ag2 were similar to the activities measured in May and June, which was unexpected.

Research in fish found an increase in EROD activity in females after spawning, while EROD activity decreased in males post-spawning (Lange *et al.* 1999). Activities in frogs were therefore expected to increase at the end of the summer in females when frogs were cycling out of breeding, as the tissue that was built up for reproduction was metabolized and frogs prepared to enter hibernation. 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 10-35 pmol/min/mg protein in adult male green frogs and 40-60 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 10-fold lesser EROD activities in juvenile frog livers than those measured in the current study (Jung *et al.* 2004). However, these lesser EROD activities may be due to the fact that the metamorphs analyzed by Jung *et al.* were younger than those analyzed in the current study. 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 which 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 is due to 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 there are site-specific environmental factors that influence metabolic activity.

Although studies have shown that EROD induction may be a useful biomarker of effect in reptiles including snakes (Hecker *et al.* in press) 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 MFO's than birds or mammals (Ertl and Winston 1998). A laboratory exposure using the known inducer PCB126 failed to produce elevated EROD activities in leopard frogs except at concentrations greater than 2.3 mg/kg (Huang *et al.* 1998). Field studies have shown that frogs accumulate persistent compounds such as PCB's, 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 the amphibian model *Xenopus laevis* was 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, although more research is required to determine if this is the case.

Significantly greater LSI values were observed in adult male green frogs from agricultural sites as compared to non-agricultural 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 Wilson 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 elevated 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 the activity of either EROD or MROD in the adult and juvenile frogs collected as a part of 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 males. Atrazine was found to induce EROD activity *in vitro* at concentrations of 2.16 μg atrazine/L (Oh *et al.* 2003), a concentration which is similar to those measured at sites sampled in the current study. A mesocosm study with carp found that atrazine induced P4501A1 gene expression at concentrations as small as 7 μg atrazine/L (Chang *et al* 2005), but an earlier study in trout 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 (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. Mammals have been reported to be more inducible than amphibians (Ertl and Wilson 1998), and the concentrations used by Hanioka *et al.* were much greater than those measured in this study. The lack of consistent relationships between atrazine concentration and EROD and MROD activities in frogs in the current study therefore may be due to a combination of generally lower inducibilities in frogs than in other organisms, exposure to relatively low atrazine concentrations in this study, and a lack of atrazine-mediated induction in frogs.

The results of the bioassay for AhR activity indicated that, with the exception of site Ag2, TEQ values at the study sites were less than 1 pg/g dry wt. EROD activities in adult female green frogs were significantly less than those measured at all the other sites, which is unexpected. AhR activity was detected in fractions II and III in the sediment extraction. Fraction II activity was likely due to the presence of PAH's; 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 is probably due 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 females 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 be due to the presence of dioxins or dioxin-like compounds and is unclear.

The lack of consistent induction or suppression patterns in EROD and MROD activities observed in frogs in this study may be due to a variety of factors. Declines in CYP1A activities have been observed in female fish that are approaching spawning, and are influenced by the health, reproductive and developmental status of fish and by environmental temperature (Whyte *et al.* 2000). Furthermore, it has been shown that low but long-term exposure to halogenated organic compounds such as dioxin 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 via competitive inhibition of enzymatic activity (Schlezinger 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, stabilizing once metamorphosis is complete (Khan *et al.* 1998). While most of the juveniles collected in the current study had completed metamorphosis, there may be still be 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 endpoints and not on gene expression of CYP1A protein levels. Thus, the variability in the enzyme data cannot be compared to 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. This is particularly important in this study, since the genes belonging to CYP1A have not been extensively identified or sequenced nor have their associated substrate selectivities been properly characterized in frogs. Consequently, while there were only significant correlations between atrazine concentrations and MROD activity, this endpoint 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 power to detect differences in EROD and MROD activity between agricultural and non-agricultural sites in adult male green frogs was relatively good (0.74 and 0.68), but the power to detect differences in green frog adult females was less than 0.30, and power to detect differences in juveniles was less than 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.

CONCLUSION

The aim of this study was to determine whether atrazine exposure was resulting in gonadal abnormalities and endocrine disruption in native ranid frog species. Examination of the gonads of male frogs both at the gross morphological and histological levels indicated that hermaphroditism and testicular oocytes occurred at low incidences at both agricultural and non-agricultural sites, regardless of atrazine exposure. The other reproductive parameters measured in this study, plasma steroid hormone concentrations and aromatase activity, similarly showed no relationship with atrazine concentrations. Aromatase activity in males was for the most part undetectable, while steroid hormone concentrations were highly variable both within and among sites in both males and females and both juveniles and adults. In addition, atrazine exposure was not related to changes in liver enzyme function that could have an effect on steroid metabolism. The results of this study therefore do not support the atrazine-mediated aromatase up-regulation hypothesis proposed by Hayes *et al.* The inter- and intra-species variability observed in many of the parameters measured in this study underscores the need for more research into seasonal and development patterns that provide the context for the interpretation of contaminant effects data.

Table 7. Site descriptions using the Cowardin *et al.* (1979) classification system

Site	Class	Dominant Plant Type	Water Regime	Soil Type	Subordinate Species
NA1	Emergent Wetland	*Carex* sp.	Permanently flooded	Organic	*Lemna minor, Alisma plantago-aquatica, Ceratophyllum demersum, Cephalanthus occidentalis, Myriophyllum sibiricum, M. verticilatum.*
NA2	Emergent Wetland	*Carex* sp.	Permanently flooded	Mineral	*Typha* sp.
NA3	Emergent Wetland	*Nuphar variegata, Nymphaea odorata*	Permanently flooded	Organic	*Typha angustifolia, Lemna minor, Eleocharis acicularis, Carex* sp., *Asclepias syriaca, Polygonum pensylvanicum, Elodea Canadensis*
NA4	Emergent Wetland	*Typha augustifolia*	Permanently flooded	Organic	*Typha latifolia, Lemna minor, Eleocharis acicularis, Scirpus acutus, Asclepias incarnata, Lythrum salicaria, Nuphar variegata, Nymphaea odorata, Elodea canadensis, Sagittaria latifolia, Polygonum amphibium, Cornus racemosa, Sparganium eurycarpum*
NA5	Emergent Wetland	*Typha sp., Myriophyllum sibiricum, M. verticilatum*	Permanently flooded	Organic	*Lemna minor, Eleocharis acicularis, Carex sp., Scirpus acutus, Asclepias incarnata, Eupatorium perfoliatum, Salix nigra, Nymphaea odorata*
Ag1	Emergent Wetland	*Typha augustifolia*	Permanently flooded	Mineral	None
Ag2[a]	Scrub-shrub Wetland	*Lemna minor*	Permanently flooded	Organic	*Cephalanthus occidentalis, Salix nigra*
Ag2[a]	Emergent Wetland	*Carex* sp.	Permanently flooded	Organic	*Lemna minor, Poeaceae sp., Salix lucida, Acer negundo, Sagittaria latifolia*

Table 7. Site descriptions using the Cowardin *et al.* (1979) classification system (cont'd)

Site	Class	Dominant Plant Type	Water Regime	Soil Type	Subordinate Species
Ag3	Emergent Wetland	*Typha augustifolia*	Semipermanently flooded	Organic	*Eleocharis acicularis*, *Carex* sp., *Impatiens capensis*, *Polygonum amphibium*, *P. lapathilfoilium*, *Bidens cernua*
Ag4	Emergent Wetland	*Lemna minor*	Permanently flooded	Organic	*Eleocharis acicularis*, *Vitis riparia*, *Salix nigra*, *Acer negundo*, *Polygonum persicaria*, *Phalaris arundinacaea*, *Urtica dioica*, *Cyperaceae* sp.
Ag5[a]	Emergent Wetland	*Typha augustifolia*	Permanently flooded	Organic	*Lemna minor*, *Eleocharis acicularis*, *Impatiens capensis*, *Asclepias syriaca*, *A. incarnata*, *Eupatorium perfoliatum*, *Vitis riparia*, *Verbena hastata*, *Lycopus americana*, *Sonchus arvensis*, *Ceratophyllum demersum*, *Lythrum salicaria*, *Solidago* sp.
Ag5[a]	Emergent Wetland	*Lemna minor*	Permanently flooded	Organic	*Typha angustifolia*, *Eleocharis acicularis*, *Alisma plantago-aquatica*, *Carex* sp., *Scirpus acutus*, *Poaceae* sp.
Ag6	Emergent Wetland	*Lemna minor*	Permanently flooded	Organic	*Alisma plantago-aquatica*, *Carex* sp., *Poaceae* sp., *Asclepias incarnata*, *Vitis riparia*, *Cephalanthus occidentalis*, *Sagittaria latifolia*, *Typha augustifolia*, *Symplocarpus foetidus*, *Fraxinus nigra*
Ag7	Forested Wetland	*Typha latifolia*	Permanently flooded	Organic	*Lemna minor*, *Eleocharis acicularis*, *Poeaceae* sp., *Impatiens capensis*, *Asclepias incarnata*, *Vitis riparia*, *Cephalantus occidentalis*, *Quercus bicolor*, *Salix lucida*, *S. nigra*, *Myriophyllum sibiricum*, *Ulmus americana*, *Acer negundo*, *Polygonum* sp.
Ag8	Emergent Wetland	*Scirpus acutus*	Permanently flooded	Mineral	*Eleocharis acicularis*, *Carex sp.*, *Populus deltoides*, *Lycopus uniflorus*, *Juncus tenuis*, *Salix* sp.

Table 7. Site descriptions using the Cowardin *et al.* (1979) classification system (cont'd)

Site	Class	Dominant Plant Type	Water Regime	Soil Type	Subordinate Species
Ag9[a]	Emergent Wetland	*Lemna minor*	Permanently flooded	Organic	*Alisma plantago-aquatica, Carex sp., Typha augustifolia, Equisetum hyemale, Acer saccharum, Populus deltoides, Salix amygdaloides, Cornus amomum*
Ag10	Emergent Wetland	*Carex sp.*	Permanently flooded	Organic	*Scirpus acutus, Asclepias incarnata, Eupatroium perfoliatum, Vitis riparia, Salix nigra, Acer saccharinum, Equisetum hyemale, Acer saccharum, Osmunda claytoniana, Salix exigua, Populus deltoides*

[a] Site was composed of two ponds; each pond is described separately.

Table 8. Land use and other matrix characteristics for study sites.

Site	Land Use Matrix	Greenbelting (Y/N)	Other Chemical Treatments	Road Adjacent to Site (Y/N)
NA1	Backyard pond	Y	None	N
NA2	Backyard pond	Y	Copper treatment for algae	N
NA3	Biological station	Y	None	Y
NA4	State game area	Y	None	N
NA5	Nature center	Y	None	N
Ag1	Backyard pond/Corn field	N	Copper treatment for algae	N
Ag2	Corn field	Y	None	Y
Ag3	Corn field	Y	None	Y
Ag4	Corn field	Y	None	N
Ag5	Corn field	Y	None	N
Ag6	Corn field	Y	None	Y
Ag7	Backyard pond/Corn field	Y	None	N
Ag8	Tree farm	Y	None	N
Ag9	Tree farm	Y	None	N
Ag10	Tree farm	Y	None	N

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