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Plasma steroid hormone concentrations, aromatase activities and GSI in ranid frogs collected from agricultural and non-agricultural sites in Michigan (USA)

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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 exist 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 summer 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 agricultural sites, but a concentration of 250 μg atrazine/L was measured in one sample from one site in 2002. Plasma steroid concentrations varied among locations. Aromatase activity was measurable in less than 11% of testes in adult males, and in less than 4% of testes in juvenile males. Median aromatase activities in ovaries of adult females ranged from 3 to 245 pmol/h/mg protein, and maximum activities were 2.5-fold greater in juveniles than in adults. Atrazine concentrations were not significantly correlated with any of the parameters measured in this study. These results indicate that atrazine does not up-regulate aromatase in green frogs in the wild, and does not appear to affect plasma steroid hormone concentrations.

Keywords: Triazine; Amphibian; Steroid hormone; Aromatase; Endocrine disruption; Field study

1. Introduction

The triazine herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) has been 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 (25.2–29.9) × 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).

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However, these maximum concentrations have a short duration and longer-term moving average concentrations seldom exceed 20 μg/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. Consequently, amphibian species may be exposed to these concentrations during their early development when they are potentially highly susceptible to endocrine disruption. This fact, coupled with widespread declines in amphibian populations, has prompted concern about the potential effects of agricultural chemicals, including atrazine, on amphibians.

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. Studies conducted under laboratory conditions in which the African clawed frog (*Xenopus laevis*), an amphibian model, was 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), while another study found effects on gonad development at 25 μg atrazine/L (Carr et al., 2003). Later studies using the same range of concentrations did not find significant effects of atrazine on gonad development of juvenile male *X. laevis* (Coady et al., 2005) or *R. clamitans* (Coady et al., 2004). However, estradiol concentrations in male *X. laevis* were significantly less in the 1 μg atrazine/L treatment when compared to the controls (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 converts androgens to estrogens. 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). This decrease has been suggested to cause 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 25 μg atrazine/L, although concentrations of estradiol, the biosynthetic end product of aromatase activity, were not measured (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 that a significant suppression of plasma T concentrations occurred only at 250 μg atrazine/L (Hecker et al., 2005a), but no effects were observed on either aromatase activity or *CYP19* mRNA expression (Hecker et al., 2005a,b). 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 demonstrated 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., 1999; Heneweer et al., 2004; Hecker et al., 2004, 2005a,b; 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).

Unlike many mammalian species, T is not the most active androgen in lower vertebrates including fish and presumably frogs. Previous studies of *X. laevis* have shown that T appears to be present at similar concentrations in both male and female frogs (Coady et al., 2005; Hecker et al., 2004, 2005a,b), indicating that T may play a different role in this species than in mammals. Little is known about the specific role of T or other androgens in amphibians, but it is possible that typical androgen-mediated processes in frogs occur through the action of specific androgens such as 11-ketotestosterone (KT). 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 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 southwestern Michigan.

2. Materials and methods

2.1. Site selection and frog sampling

Site selection and frog collection procedures used in this study have been described elsewhere (Murphy et al., in press). 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) (Fig. 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 (Fig. 1). Detailed site descriptions and atrazine concentrations are presented by Murphy et al. (in press) and Murphy (2005).

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

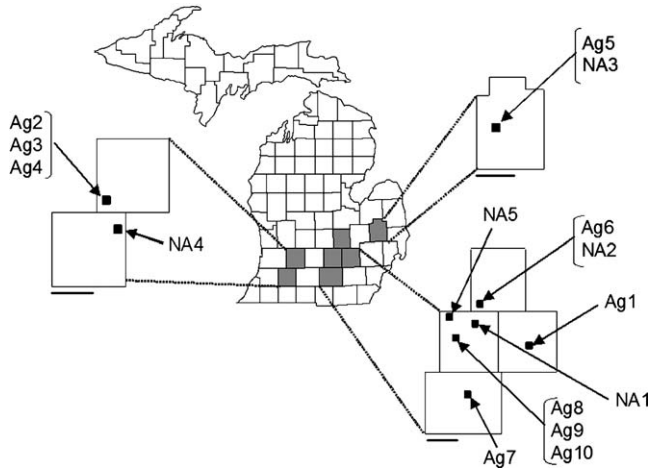

Fig. 1. Site locations in Michigan. Sites were located in three major regions: near Kalamazoo (KZ), in the area around Michigan State University (MSU) and near Lapeer (LPR), in both agricultural ("Ag") and non-agricultural areas ("NA"). Bars represent a distance of 20 km.

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 parameters.

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 500 mg/L MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO, USA), and each frog was weighed and snout-vent length (SVL) was measured. All procedures involving animals were approved by and conducted in accordance with the policies of the All-University Committee on Animal Use and Care at Michigan State University under animal use permit #01/04-035-00.

2.2. Blood and tissue sampling

Blood was taken from each frog using a heparinized syringe (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 4-h time window at approximately the same time of day to reduce the effects of confinement stress and diurnal cycling on hormone levels. A longitudinal 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 removed and flash-frozen in liquid nitrogen for determination of aromatase activity. The remaining gonad was left in the body and the entire frog was preserved in Bouin's solution (Sigma) for subsequent histological analysis (Murphy et al., in press). 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 were not collected from frogs from both agricultural and non-agricultural sites for this reason, and the %-not collected was no greater than 18.5% of males at any one site; this maximum percentage represents gonads not collected from 10 out of 54 male frogs at site Ag2 in 2002 and 5 out of 27 male frogs at site NA4 in 2003. 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 (Eq. (1)):

$$\text{GSI} = (\text{gonad weight/body weight}) \times 100 \quad (1)$$

2.3. 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 (9.3 mM NaH_2PO_4, 40.5 mM Na_2HPO_4, 61.8 μM thimerosal, 1 g/L gelatin, pH 7.6). T, E2 and KT concentrations were measured using competitive enzyme-linked immunosorbent assays (ELISAs) developed by Cuisset et al. (1994) with modifications described by Hecker et al. (2002); because KT concentrations could not be confirmed analytically, measurement of KT was termed "KT-immunoreactive" (KT-IR). 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 antisera were obtained from D.E. Kime (University of Sheffield, Sheffield, UK). The T antibody cross-reacted with 11β-hydroxytestosterone (13.8%), KT (12.2%) and 5α-dihydrotestosterone (DHT; 4.0%); the KT antibody cross-reacted with 11β-hydroxytestosterone (2.2%) and T (1.3%; Kime and Manning, 1982; 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 DHT (0.1%); all other steroids cross-reacted with the E2 antibody at less than 0.1%. In addition, because DHT is known to be a potent androgen in fish (Kime, 1993), specificity of the KT-AB to KT-IR was verified by spiking experiments with DHT. 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 the small quantities of plasma available.

2.4. 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 μL of the homogenate was used in the assay. Adult and juvenile gonads were homogenized in 900 and 600 μL assay buffer, respectively. Reagents were added to the homogenate as follows: 54 nM ^{3}H-androstenedione (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 competitive tests with non-labeled androstenedione or fadrozole, a known aromatase inhibitor, as described by Hecker et al. (2005a). Addition of 7.5 μL of 5.6×10^2 μM 4-androsten-4-ol-3,17-dione or 5 μM fadrozole reduced tritiated water formation to the levels found in the tissue blanks, demonstrating that the activity being measured was specific for aromatase. Protein concentrations were determined using the Bradford assay with bovine serum albumin (Sigma) as the standard (Bradford, 1976). The lowest detectable activity for the tritium-release assay was 0.58 fmol/h/mg prot.

2.5. 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 natural log (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 non-parametric Kruskal–Wallis tests. General linear models were used to test for significant relationships between atrazine concentrations and median GSI values, hormone concentrations and aromatase activities. If significant linear relationships were found, Pearson pair-wise (product moment) correlations were used to test for relationships between these parameters. 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.

3. Results

All of the measured parameters exhibited significant variability within and among sites (Figs. 2–7). Due to the larger sample size obtained for green frogs, statistical analyses were conducted only on this species. However, to attempt to determine interspecies differences in the measured parameters, comparisons with bullfrogs and leopard frogs were made whenever possible.

3.1. GSI and hormone concentrations

3.1.1. Males

With the exception of GSIs, all parameters measured showed considerable variation both among and within sites during both sampling seasons (Figs. 2 and 3). 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-IR concentrations, as well as the ratios, E2/T and KT/T of adult male green frogs were significantly different among all locations (Fig. 2 and Table 1). 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-IR 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 1). However, power analysis indicated that the power to detect a four-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 (Fig. 3 and Table 1). 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-IR 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 the same 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 1). Power analysis indicated that a 1.5-fold difference in GSI could be detected ($\beta = 0.933$), but the ability to detect four-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 greater in agricultural compared to non-agricultural sites (Fig. 6 and Table 1). The power to detect differences in E2 concentrations was found to be less than 0.10. The greatest median T and E2 concentrations and the greatest and most variable T concentrations were measured in juvenile green frogs from site Ag9 (T = 1.42 ng/mL, E2 = 0.28 ng/mL, Fig. 6), while the greatest and most variable E2 concentrations overall were measured in juvenile green frogs from site NA5. 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-IR concentrations were generally greater in frogs collected in 2003.

Lesser GSI values were measured in adult male bullfrogs than in adult male green frogs at all sites where both

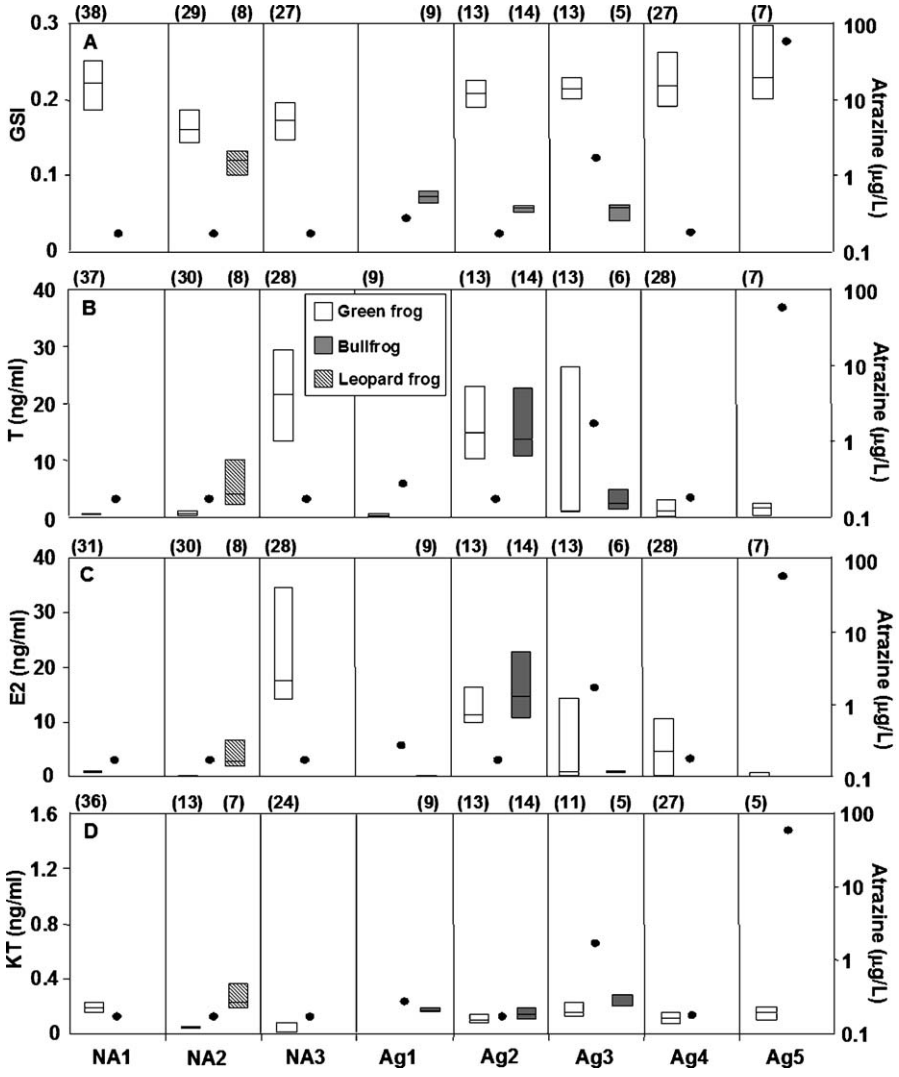

Fig. 2. GSI (A) and T (B), E2 (C), and KT (D) concentrations measured in adult male frogs in 2002. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. The numbers in parentheses above the graphs represent sample size for each species. Points represent the third quartile of the atrazine concentrations measured in water from the sites. Results of site and land use comparisons are in Table 1.

species were collected in both years of the study. Plasma concentrations of T and KT-IR 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.

3.1.2. Females

Adult females in 2002 differed significantly among sites in terms of GSI, T, E2, E2/T, KT-IR and KT/T (Fig. 4 and Table 1). 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-IR concentration was measured at site Ag3 (0.19 ng/mL). E2/T, KT-IR and KT/T were significantly different at agricultural sites compared to non-agricultural sites, with greater values at agricultural sites (Table 1). The power to detect differences in GSI, T and E2 in 2002 was less than 0.20.

In 2003, E2, KT-IR and KT/T differed significantly among sites, but none of the measured parameters differed between agricultural and non-agricultural sites (Fig. 5). 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-IR 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 (Fig. 6 and Table 1). Of these parameters, only T concentrations differed between agricultural and non-agricultural sites, with greater concentrations measured at agricultural sites (Table 1). 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.

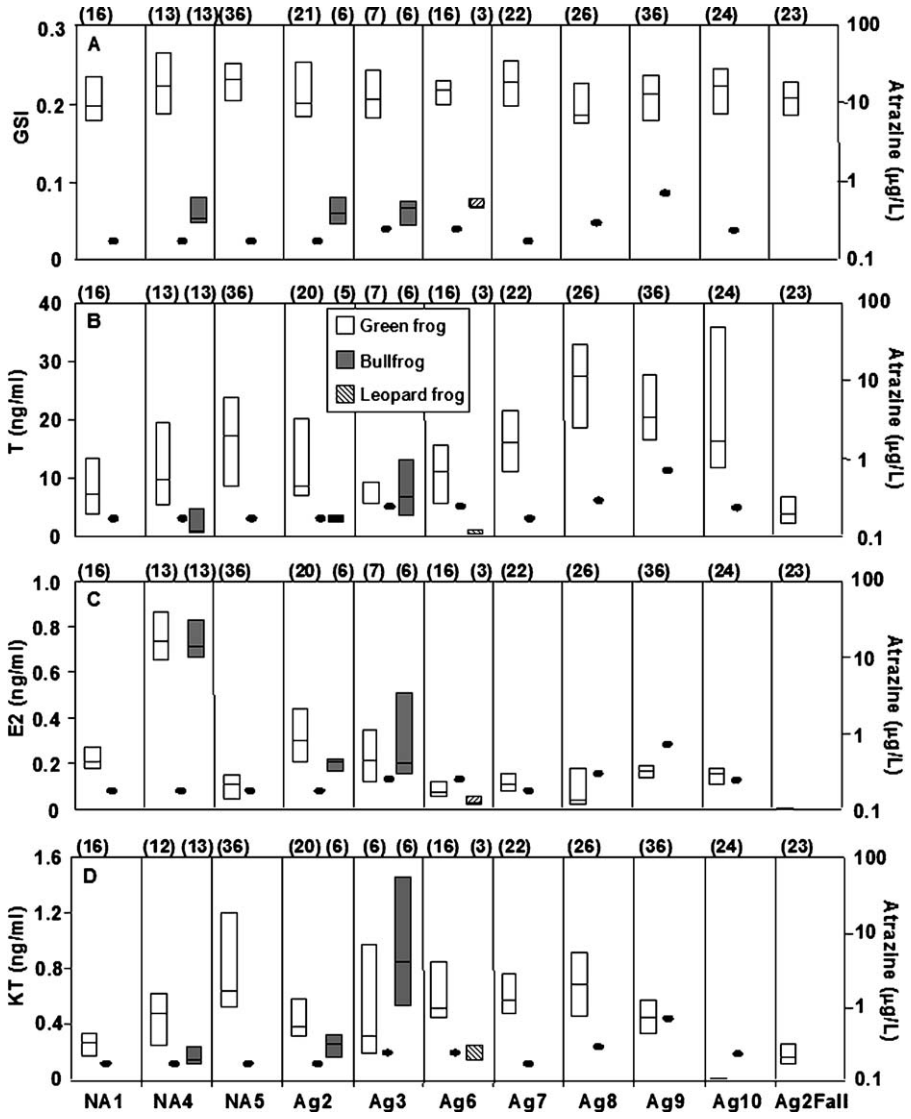

Fig. 3. GSI (A) and T (B), E2 (C), and KT (D) concentrations measured in adult male frogs in 2003. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. The numbers in parentheses above the graphs represent sample size for each species. Points represent the third quartile of the atrazine concentrations measured in water from the sites. Results of site and land use comparisons are in Table 1.

3.2. Aromatase activities

3.2.1. Males

Aromatase activities were less than the lowest measured activity in the assay (0.58 fmol/h/mg prot) in most of the testes analyzed. For statistical analysis, 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/h/mg protein for adults and juveniles, respectively. The same values were used for both males and females in each age class.

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 aromatase activities greater than the detection limit of the assay; of these, only three had activities greater than 20 fmol/h/mg prot. The maximum measured activities were found in two frogs from site Ag2; 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. 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.

3.2.2. Females

Aromatase activities in ovaries of female green frogs were much greater than those measured in the testes of males. How-

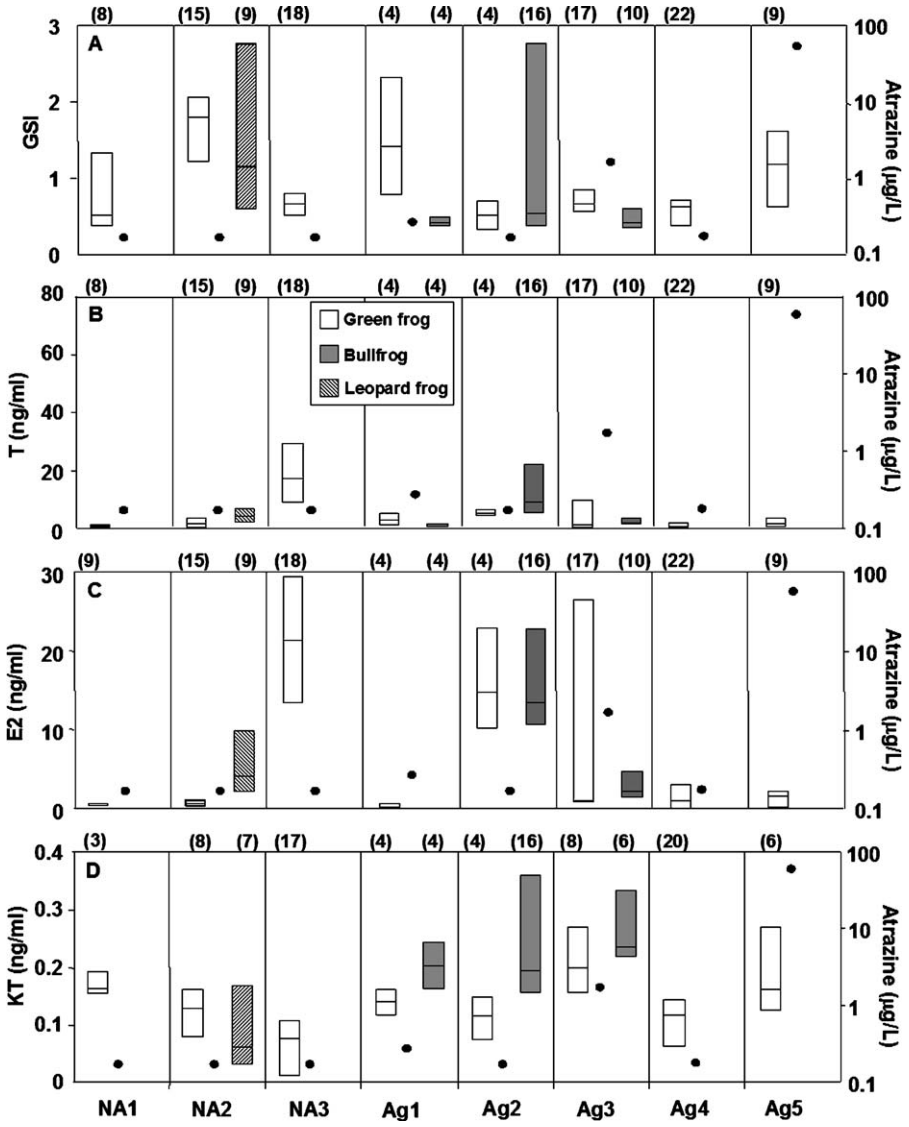

Fig. 4. GSI (A) and T (B), E2 (C), and KT (D) concentrations measured in adult female frogs in 2002. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. The numbers in parentheses above the graphs represent sample size for each species. Points represent the third quartile of the atrazine concentrations measured in water from the sites. Results of site and land use comparisons are in Table 1.

ever, aromatase activity was still less than the detection limit of the assay in 7 of 136 adult females (5.1%) and 55 of 124 juvenile females (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 (Fig. 7). 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 1). 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 (Fig. 7 and Table 1). 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 (Fig. 7 and Table 1).

3.3. Atrazine correlations

Correlations were determined between atrazine concentrations measured approximately 4 weeks before collection of the frogs at a site (T-28) and those measured at the time of sampling (T-0) and the hormone concentrations, their ratios and aromatase activities. Atrazine concentrations were not correlated with GSI, T, E2, E2/T, KT-IR, KT/T or aromatase activity in adult male green frogs or juvenile female green frogs collected in 2002 or 2003.

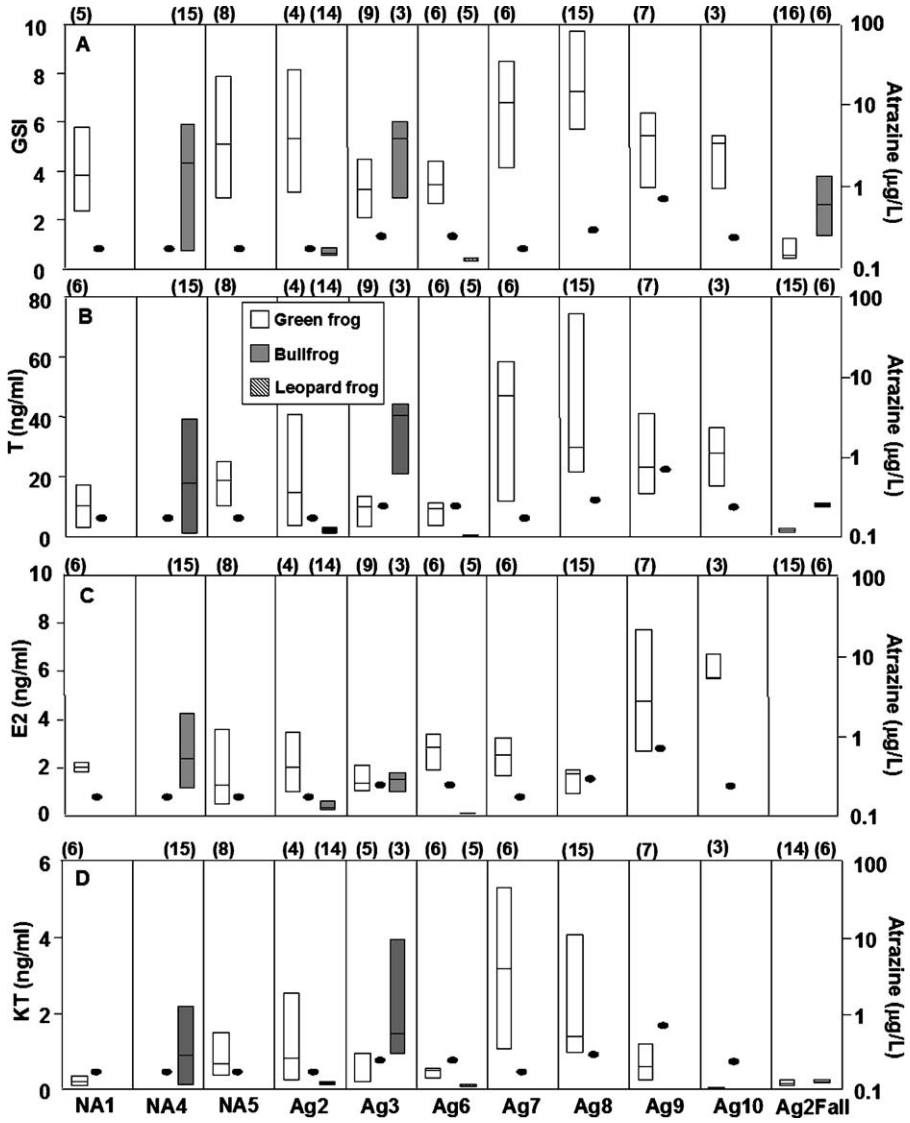

Fig. 5. GSI (A) and T (B), E2 (C), and KT (D) concentrations measured in adult female frogs in 2003. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. The numbers in parentheses above the graphs represent sample size for each species. Points represent the third quartile of the atrazine concentrations measured in water from the sites. Results of site and land use comparisons are in Table 1.

4. Discussion

4.1. Seasonal patterns

The variability observed in plasma hormone concentrations between the two sampling years can be largely 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 as the other parameters measured when comparing frogs collected in late summer 2002 with those collected in early summer 2003. 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 followed by a decrease in

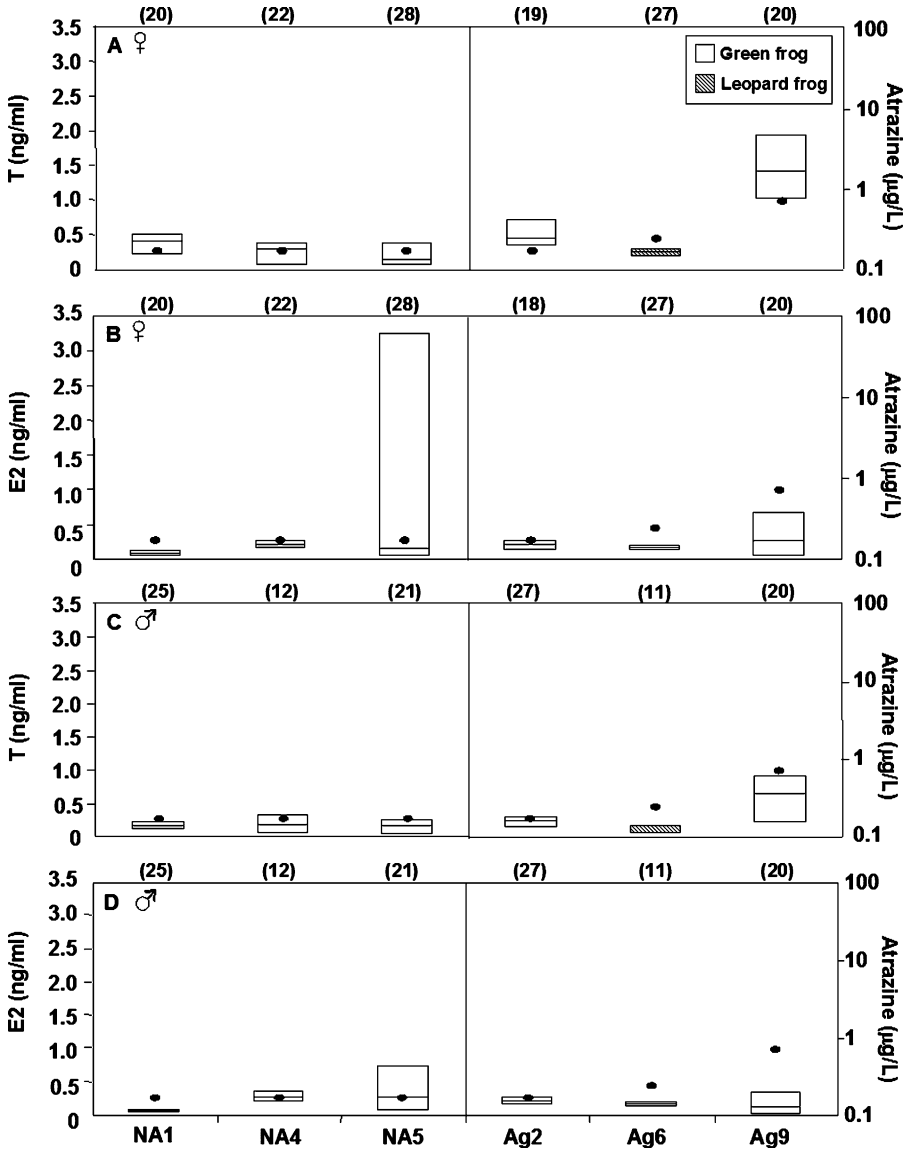

Fig. 6. Juvenile male T (A) and E2 (B) and juvenile female T (C) and E2 (D) concentrations in 2003. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. The numbers in parentheses above the graphs represent sample size for each species. Points represent the third quartile of the atrazine concentrations measured in water from the sites. Results of site and land use comparisons are in Table 1.

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 (Licht et al., 1983). This fluctuation is consistent with a strategy where breeding can occur more than once; however, the sampling scheme in the current study was not designed to 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 developmental processes such as growth. 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.

GSI values in female frogs were highly variable both within and among sites, likely reflecting differences in breeding time and the number of mating events. Previous research has shown that GSI in female bullfrogs peaked once in May–June, and also varied between individuals (Licht et al., 1983). In *R. esculenta*, GSI values peaked in April, declined sharply, and then

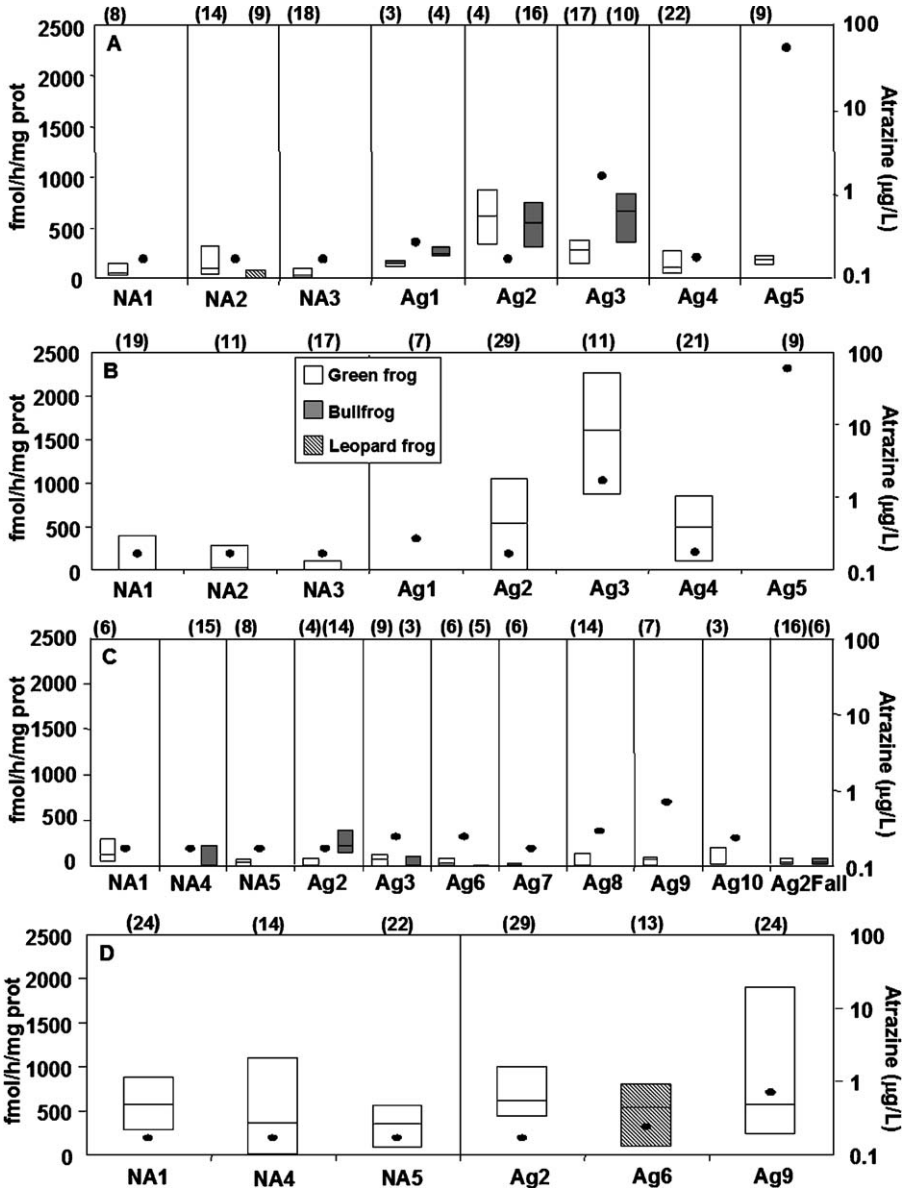

Fig. 7. Aromatase activities measured in female frogs: 2002 adults (A) and juveniles (B); 2003 adults (C) and juveniles (D). Greater activities were measured in juvenile than adult frogs. The horizontal line on each bar represents the median, and bar length represents the middle-50% of the data. The numbers in parentheses above the graphs represent sample size for each species. Points represent the third quartile of the atrazine concentrations measured in water from the sites. Results of site and land use comparisons are in Table 1.

began to increase again during the September "recovery" period (Mosconi et al., 1994; Polzonetti-Magni et al., 1998).

Comparisons between green frogs and 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-IR in late summer 2002 than in early summer 2003. These greater concentrations may indicate that leopard frogs begin to synthesize hormones to initiate gonadal maturation 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. Studies with *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 may not require this species to begin preparing for the next season before entering hibernation. Research on bullfrogs in California, USA found that females began building up ovarian tissue in February, while GSI values were greatest in May

Table 1
Inter-site and land use comparison *p*-values for the measured parameters in green frogs using Kruskal–Wallis and Mann–Whitney *U* tests

	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

[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.

when egg laying was at its peak, and then declined until August, when the cycle started again (Licht et al., 1983); this pattern is consistent with the GSI values measured in green frogs in the current study, which were as much as three-fold greater in early summer 2003 than in late summer 2002. A high degree of asynchrony and variability similar to that seen in the current study was also found in bullfrogs (Licht et al., 1983). At this point, however, the pattern of gonad growth and reabsorption in green frogs is unknown.

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 years 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 frogs sampled from the site at which the greatest aromatase activities were observed, Ag9, did not have greater concentrations of E2 or lesser concentrations of T than juvenile females from other sites (Figs. 6 and 7). 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. E2 is known to function in reproductive development in male mammals (Sharpe, 1998). In goldfish (*Carassius auratus*), studies have found that T can stimulate growth hormone (GH) mRNA expression in juveniles (Huggard et al., 1996), while E2 exposure increased GH concentrations in adults (reviewed by Peng and Peter, 1997). GH is known to play a role in growth and food intake in *X. laevis* (Huang and Brown, 2000). It is therefore possible that elevated aromatase activity in juvenile frogs is related to growth, but more research into this question is needed.

Juvenile frogs were sampled in July in both years of the study, but differences in aromatase activities in females between agricultural and non-agricultural sites were greater in 2002 than in 2003. 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, possibly through effects on habitat quality. For example, hotter temperatures could result in greater water evaporation rates, which could in turn result in greater tadpole growth rates to avoid the possibility of desiccation at low water levels. 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-IR in late summer 2002, but KT-IR 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-IR 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-IR measured in the current study showed the same pattern, peaking in the spring at greater concentrations in females. However, similar concentration patterns between T and KT-IR would be expected if DHT

accounted for some of the KT-IR concentrations measured in this study, but these similar 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. However, the relatively great KT-IR concentrations measured in female frogs makes it unlikely that KT is an active androgen in frogs.

4.2. Atrazine effects

Atrazine concentrations were not correlated with the majority 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. However, Hayes et al. (2002) found effects of atrazine on plasma T in adult frogs at concentrations of 25 μg/L, a concentration that is more than 10-fold greater than those reported during this study with the exception of two measurements at one site.

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 male frogs analyzed. Three adult (one green frog each from sites Ag2 and NA5, one bullfrog from Ag2) and one juvenile male frog (from site Ag2) 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 (TOs) in the testis, which produce estrogens, as hypothesized by Hayes et al. (2002). However, in a parallel study, the gonads that were preserved in the frogs were analyzed histologically for the presence of TOs, and none were found in the other testes of these two frogs (Murphy et al., in press). A comparison of hormone parameters and the number of TOs indicated no correlation between these endpoints for any parameter (Spearman $R < 0.100$). This lack of correlation, as well as the fact that there were no TOs 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 TOs were present in the individual testes analyzed for aromatase, as studies in *X. laevis* have found that TOs can occur in either one or both testes (Jooste et al., 2005). Two of the three adult male frogs with the greatest activities were collected from the same site (Ag2), 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 for 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, 2005), as well as no effect of atrazine on *CYP19* gene expression and aromatase activity (Hecker et al., 2005).

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 two-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 parameters measured in this study. These differences, however, were not consistent among parameters, sex, age class, or season, and therefore may be due to factors other than atrazine, including 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., in press). However, the possibility that other contaminants were present at the sites that could affect hormone concentrations cannot be excluded. 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 conflict-

ing results, with some species appearing to be more successful in agricultural habitats than others (Knutson et al., 2004; Kolozsvary and Swihart, 1999; Gray et al., 2004). A study of the *R. esculenta* complex found that T concentrations were significantly less in frogs from agricultural sites compared to pristine sites, while E2 concentrations were greater at agricultural sites (Mosconi et al., 2005). In contrast, the current study found significantly greater T concentrations in adult and juvenile males and in juvenile females at agricultural sites, and no significant difference in E2 concentrations between agricultural and non-agricultural sites. However, 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.

5. 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. Measured plasma hormone levels were highly variable across sites in both males and females.

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