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Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas

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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 hypothesised 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 male 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

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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.

Keywords: Triazine herbicides; Amphibian; *Rana clamitans*; Field study; Testicular oocyte; Hermaphrodite

1. 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 30,100 metric tonnes of active ingredient were applied in the US, 75% of which was applied to corn (US EPA, 2003). Between 1998 and 2002, 815 tonnes 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 that 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 days, depending on soil and environmental conditions, while the half-life of atrazine in the aquatic environment ranges from 41 to 237 days (Giddings et al., 2004). One study of atrazine biotransformation in anaerobic wetland sediment found that the half-life of the parent compound was 224 days (Chung et al., 1996), while a later study found that the half-life was only 38 days (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, 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, 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.

2. Materials and methods

2.1. 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) (Fig. 1). Sites adjacent to corn fields were classified as “agricultural”, while those in the same general area that did not receive direct runoff from corn fields, either

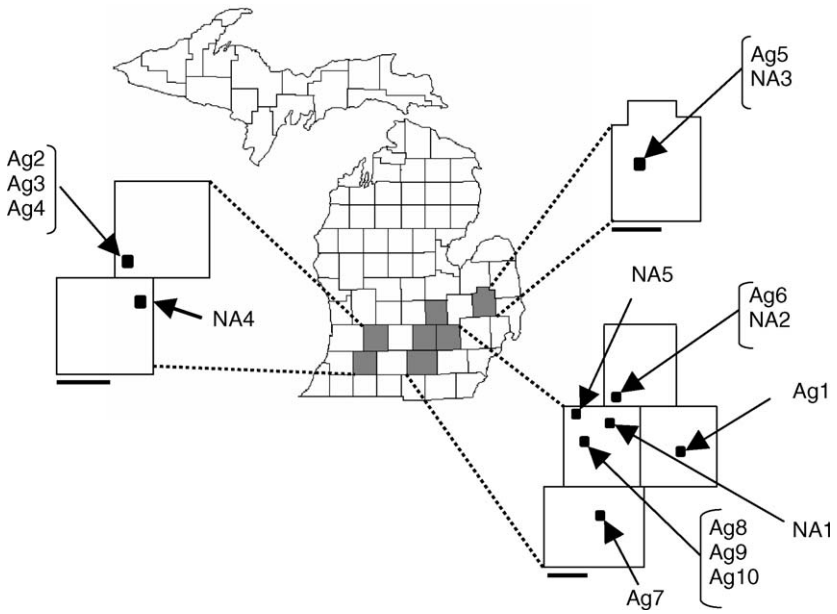

Fig. 1. Site locations in Michigan for frog collections. Sites were located in three major regions (left-to-right on the map): near Kalamazoo, in the greater Lansing area and near Lapeer, in both agricultural ("Ag") and non-agricultural areas ("NA"). Bars represent a distance of 20 km.

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 approximately monthly from May to September to characterize the concentrations of atrazine and other agricultural chemicals. Samples were taken in 1 L water-methanol-rinsed amber glass I-Chem bottles (Fisher Scientific, Hampton, NH, USA). Samples were taken approximately 10 cm below the water's surface, and mesh was used to exclude as much organic matter as possible from the samples. 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) of the ELISA 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 randomly 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 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.

2.2. 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). Male frogs typically arrive at breeding ponds in the late spring and early summer, while females arrive later once males have established territories (Martof, 1953). 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 from egg masses laid earlier that summer. 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.

Both juvenile and adult frogs were collected during 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 #01/04-035-00. 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 13 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 for aromatase measurement (Murphy et al., in press). 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 not disturbed 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 that is observable at the gross morphological level, while the term "testicular oocytes" was used to describe cases where single or multiple oocytes were found in the testes of male frogs at the histological level.

2.3. 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 TOs, using an Olympus BX41 TF microscope (Olympus, Melville, NY). TOs were enumerated and reported as the number of TO per animal. TO stages were determined using the classification system of Dumont (1972). Stage II TOs were characterized by the presence of nucleoli and lampbrush chromosomes, whereas stage III TOs were characterized by an overall increase in cell size, and the presence of yolk proteins in the lighter-staining cytoplasm (Dumont, 1972). A male gonad that contained more than 30% TOs was classified as an ovotestis.

2.4. 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 between atrazine concentrations and environmental metrics. Significant inter-year differences in mean atrazine concentrations were determined using non-parametric Mann–Whitney *U*-tests. 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 the rank correlation model described by Spearman. 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

3.1. 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 days. Atrazine concentrations at non-agricultural sites were all below the LOQ of 0.17 μg atrazine/L in 2002 and ranged from non-detectable 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 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

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

Concentrations were determined by triazine ELISA, for which the limit of quantification was 0.17 μg atrazine/L.

[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

Table 2
Atrazine concentrations measured in water samples collected at study sites in 2003

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

Concentrations were determined by triazine ELISA as described above.

[a] Site Ag3 dried up in late summer.

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 Murphy (2005).

3.2. 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 ($p > 0.995$, d.f. = 8, χ^2-test) or in adults and juveniles in 2003 ($p > 0.950$, d.f. = 10 and $p > 0.500$, d.f. = 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.

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

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

3.3. Gross gonadal morphology

The site-specific 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 15 study sites, and represented an overall incidence of 0.54% in 2002 and 0.29% in 2003. Hermaphroditic individuals were all green frogs (Figs. 2 and 3). In 2002, one juvenile hermaphrodite was found at each of sites NA3 and Ag4 (2.1 and 2.0% site incidence, respectively). The juvenile from site NA3 had a normal testis on one side and an ovotestis on the other (Fig. 3E), while the juvenile from site Ag4 had a complete testis on one side and a complete ovary on the other (Figs. 2C and 3D). No hermaphroditic adults were collected in 2002. In 2003, one adult hermaphrodite was collected from site Ag7 (3.4% site incidence) (Figs. 2D and 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% site incidence), and in two juvenile male frogs collected at site NA5 in 2003 (7.1% site incidence). A male bullfrog with only one testis was collected from site Ag5 in 2002.

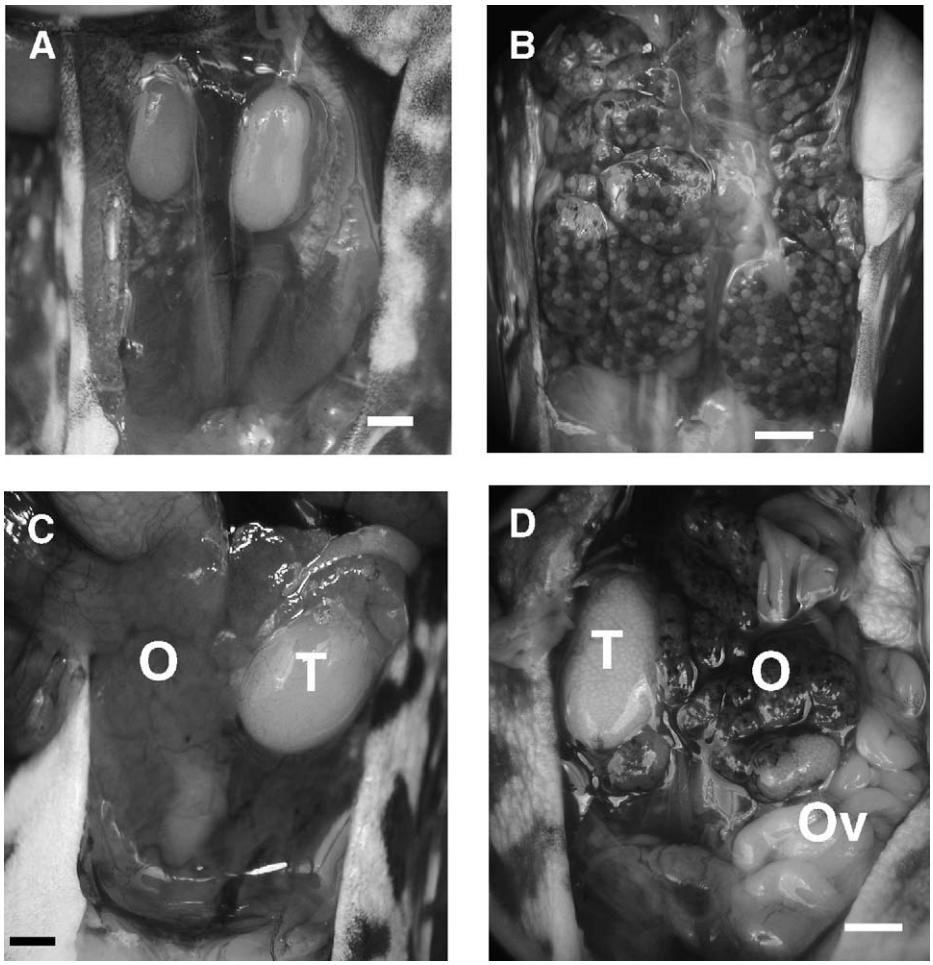

Fig. 2. Gonadal gross morphology of ranid frogs. (A) Juvenile male with normal morphology. Note the slight difference in gonad size, with the right larger than the left; this morphology appears to be typical in ranids. Bar represents 2 mm. (B) Adult female with normal morphology; bar represents 5 mm. (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.

3.4. Gonad histology

Testicular oocytes 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 (Figs. 3 and 4). TOs were discrete structures separate from the surrounding testicular tissue (Fig. 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 (Fig. 3B). Juvenile leopard frogs had the greatest overall incidence of TO (Fig. 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 (Fig. 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 (Fig. 3F).

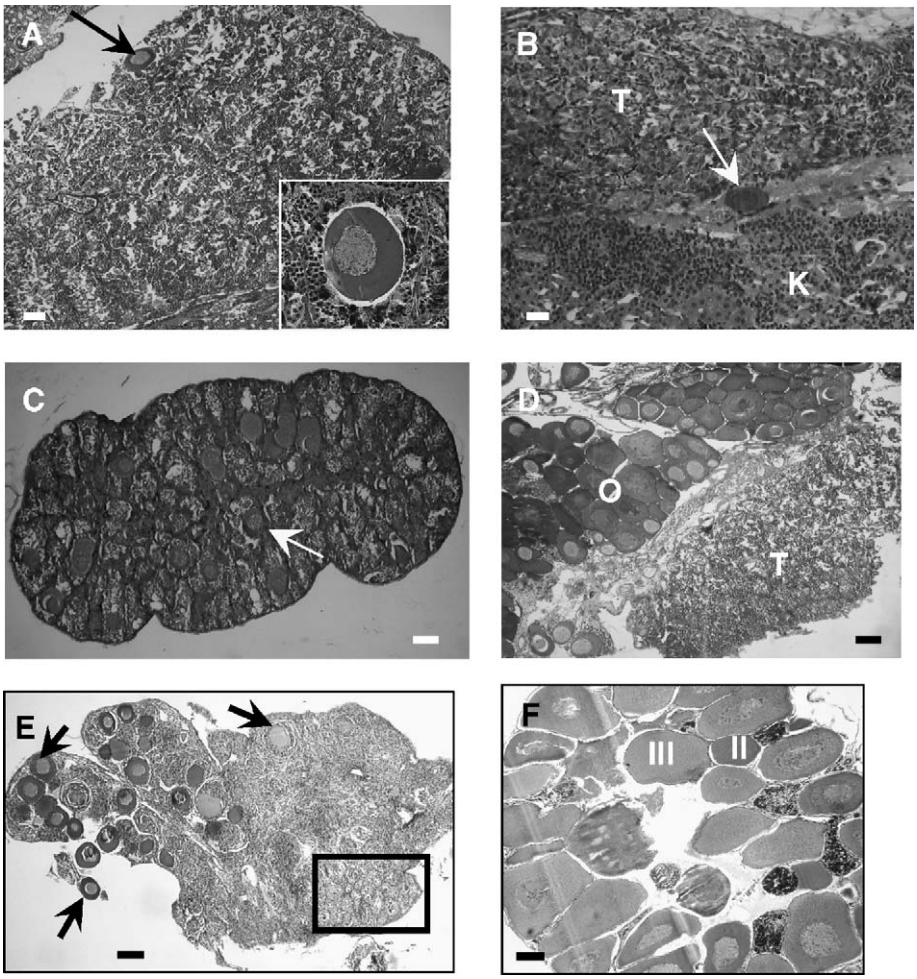

Fig. 3. Gonad histology of ranid frogs. (A) Green frog testis with a single oocyte (arrow). Inset shows typical stage II oocyte morphology. Bar represents 50 μm. (B) Green frog with an extra-testicular oocyte lying between the testis (T) and kidney (K) (arrow). Bar represents 50 μm. (C) A leopard frog 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 Fig. 2C) 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 Fig. 2D). Note that this frog has both stages II and III oocytes. Bar represents 100 μm.

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

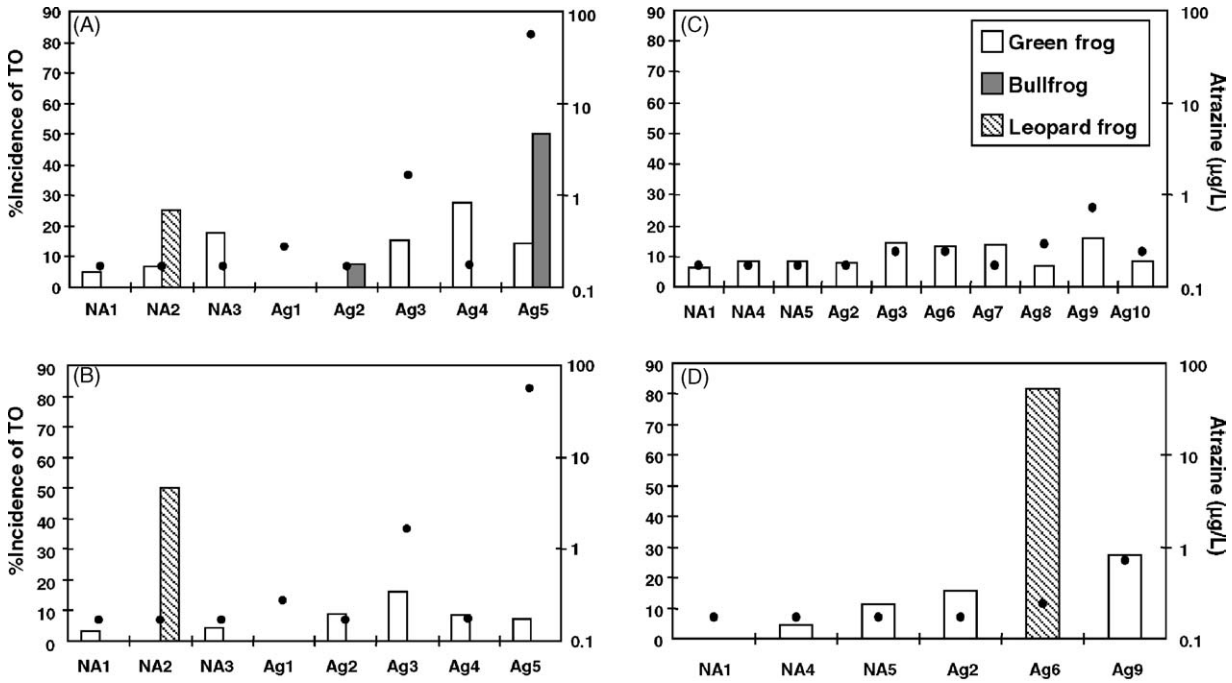

Fig. 4. Incidence of testicular oocytes in adult and juvenile ranid frogs. Incidences were determined in adults (A) and juveniles (B) collected in 2002 and in adults (C) and juveniles (D) collected in 2003. Points represent the third quartile of the atrazine concentrations measured at the sites. TO incidence was significantly greater at agricultural sites than at non-agricultural sites in juveniles collected in 2003 ($p < 0.005$, d.f. = 2, χ^2-test), and was correlated with maximum atrazine concentrations (Spearman $R = 0.820$).

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

Site	Adult male, *n*	Sex ratio (M:F)	#Frogs with TOs	#TOs per animal	Species	Juvenile male, *n*	Sex ratio (M:F)	#Frogs with TOs	#TOs per animal	Species
NA1	38	39:10	2	1	*R. clamitans*	31	32:23	1	1	*R. clamitans*
NA2	30	30:15	2	2, 15	*R. clamitans*	22	24:12	0	0	*R. clamitans*
NA2	8	8:11	2	1, 2	*R. pipiens*	4	4:1	2	1, 8	*R. pipiens*
NA3[b]	28	28:18	5	1, 1, 1, 3, 27	*R. clamitans*	23	24:23	1	5	*R. clamitans*
Ag1	9	9:4	0	n/a	*R. catesbeiana*	4	5:11	0	0	*R. clamitans*
Ag2	13	13:4	0	n/a	*R. clamitans*	44	54:40	4	1, 1, 4, 12	*R. clamitans*
Ag2	13	14:16	1	95	*R. catesbeiana*	–	–	–	–	–
Ag3	13	13:18	2	3, 32	*R. clamitans*	25	28:13	4	1, 1, 1, 7	*R. clamitans*
Ag3	6	6:10	0	n/a	*R. catesbeiana*	–	–	–	–	–
Ag4[b]	29	28:22	8	1, 2, 2, 4, 5, 6, 7, 33	*R. clamitans*	24	23:24	2	1, 11	*R. clamitans*
Ag5	7	7:9	1	1	*R. clamitans*	14	14:9	1	1	*R. clamitans*
Ag5	2	2:1	1	76	*R. catesbeiana*[a]	–	–	–	–	–

A dash indicates that no frogs of that age class were collected at the site.
[a] This frog had only one testis.
[b] One hermaphroditic juvenile was collected at this site.

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

Site	Adult, *n*	Sex ratio (M:F)	#Frogs with TO	#TO per animal	Species	Juvenile, *n*	Sex ratio (M:F)	#Frogs with TO	#TO per animal	Species
NA1	16	16:6	1	81	*R. clamitans*	20	20:25	0	n/a	*R. clamitans*
NA4	12	13:1	1	1	*R. clamitans*	24	27:15	1	3	*R. clamitans*
NA4	12	13:15	0	n/a	*R. catesbeiana*	–	–	–	–	
NA5	33	36:8	3	1, 4, 4	*R. clamitans*	27	28:23	3	4, 5, 8	*R. clamitans*
Ag2	37	44:20	3	1, 1, 2	*R. clamitans*	19	19:28	3	1	*R. clamitans*
Ag2	7	8:20	0	n/a	*R. catesbeiana*	–	–	–	–	–
Ag3	7	7:9	1	1	*R. clamitans*	–	–	–	–	–
Ag3	6	6:3	0	n/a	*R. catesbeiana*	–	–	–	–	–
Ag6	15	16:6	2	6, 7	*R. clamitans*	27	28:14	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	4:5	0	n/a	*R. pipiens*	–	–	–	–	–
Ag7[a]	22	26:15	3	1, 1, 1	*R. clamitans*	–	–	–	–	–
Ag8	14	36:7	1	2	*R. clamitans*	–	–	–	–	–
Ag9	32	24:3	5	1, 1, 2, 3, 5	*R. clamitans*	22	22:24	6	1, 1, 2, 2, 2, 6	*R. clamitans*
Ag10	24	22:6	2	1, 5	*R. clamitans*	–	–	–	–	–

A dash indicates that no frogs of that age class were collected at the site.
[a] One hermaphroditic adult was collected from this site.

4. 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 86 days (Seybold et al., 2001) and 48 days (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 Schroder, 2005), and inhibited sperm motility in *Xenopus laevis* by approximately 12% at concentrations of 31 μg/L (Christensen et al., 2004). In the current 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, the effects of Zn 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 effects of As in amphibians have 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.

The frogs collected over 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. This variability in reproductive stage makes it difficult to assess contaminant effects on reproductive processes, but also makes it unlikely that an entire adult population would be affected by an endocrine-modulating compound, as only a small percentage of individuals would be at the affected or sensitive reproductive stage at any given time. In the case of a pesticide such as atrazine that has a relatively short half-life in the environment, the likelihood of effects on the population level would be even more reduced.

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 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 hermaphroditism 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 TOs (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; this problem of a lack of common terminology is reviewed by Hecker et al. (in press). 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 (*Platichthys flesus*; 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 major question surrounding atrazine is whether it affects the sexual development of 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. Hermaphroditic individuals have been observed historically in healthy frog populations and it is unlikely that these are 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. Historical observations of hermaphroditism have also been made in other ranid species, including *R. pipiens*, *R. esculenta*, *R. sylvatica* and *R. catesbeiana* (reviewed in Hecker et al., in press). More recent studies of genetics and ontology have shown that amphibians in general, and frogs in particular, possess a great deal of plasticity in their 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). In this context, the low incidence of hermaphroditic individuals collected in this study (less than 1% of all frogs collected in each year) 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 sexual characteristics. This frog also demonstrated morphology characteristic of a functional female, having both stage II (pre-vitellogenic) and stage III (early vitellogenic) oocytes. In a parallel study, 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) (Murphy et al., in press). 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 oocyte development and maturation in this frog is therefore unclear at this stage, but from an endocrinological perspective, the parallel occurrence of potentially functional testicular and ovarian tissue deserves further investigation.

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 non-agricultural sites, and TOs were observed in both adults and juveniles in all three species collected. 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. Given the fact that juveniles were collected at the same time in both years of the study, as well as the fact that a greater range of atrazine concentrations was measured in 2002 when no correlation with TO incidence was observed, this inconsistent result makes it unlikely that the TOs found in male frogs were related to atrazine exposure, including the relatively great concentrations measured during at least part of the tadpole development period in 2002.

The lack of consistent correlation between TO incidence and atrazine concentration, coupled with the low incidence of hermaphroditism observed in the green frogs collected, is in contrast to previous studies that suggested that concentrations as small as 0.1 μg atrazine/L induced gonadal abnormalities, such as discontinuous testes, TOs and hermaphroditism in the African clawed frog (*X. laevis*) and in leopard frogs (*R. pipiens*) (Hayes et al., 2002, 2003). The occurrence of TOs in frogs in one of these studies was suggested to be caused by exposure to atrazine and was never observed in unexposed frogs (Hayes et al., 2002). A field study of leopard frogs conducted in 2002 found TO in 92% of the males in one population collected from a reference pond in Wyoming (Hayes et al., 2003), an incidence similar to that observed in juvenile *R. pipiens* in the current study. 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 atrazine exposure on testicular or laryngeal development at concentrations smaller than 10 μg atrazine/L, and found a TO incidence of 2–3% at both corn-growing and non-corn-growing sites (Smith et al., 2005). Furthermore, no effects of atrazine on gonadal development and the presence of TOs 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. (2005) 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. 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 maximum of 5 TOs per animal while those males analyzed at metamorphosis had a maximum of 58 TOs per animal. The authors hypothesised that the presence of TOs 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 comparable numbers of TOs, but it may occur in leopard frogs where greater numbers of oocytes were observed in juveniles when compared to adults.

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 indi-

cated that leopard frogs were more sensitive than wood frogs to the effects of these compounds (MacKenzie et al., 2003), but further research into this issue is needed. Adult leopard frogs collected for this study had no more than two 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 currently are not fully 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 hypothesised 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. Abnormalities of this kind were not observed in the male frogs collected for the current study, and to date the effects of TOs on reproduction in frogs are unknown. However, a study in South Africa found no differences in population structure in *X. laevis* inhabiting ponds in corn-growing and non-corn-growing areas, and found robust populations at both site types (Du Preez et al., 2005). 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 consistent 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 collected 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 $\beta = 0.8$. 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 a population large enough 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 struc-

ture 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.

5. Conclusion

This study has failed to demonstrate a consistent change in the incidence of gonadal abnormalities at both the gross morphological and histological levels in *R. clamitans*, *R. catesbeiana* and *R. pipiens* collected in agricultural and non-agricultural environments in central Michigan. Testicular oocyte incidence was associated with exposure to atrazine in juveniles in 2003, but in no other age class or sampling year. Nevertheless, it is clear that there are marked differences in the incidence of gonadal abnormalities in various ranid species and understanding the factors controlling gonadal development remains a fundamental question in amphibian biology.

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