# Atrazine-Induced Hermaphroditism at 0.1 ppb in American Leopard Frogs (*Rana pipiens*): Laboratory and Field Evidence

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**Atrazine is the most commonly used herbicide in the United States and probably the world. Atrazine contamination is widespread and can be present in excess of 1.0 ppb even in precipitation and in areas where it is not used. In the current study, we showed that atrazine exposure (≥ 0.1 ppb) resulted in retarded gonadal development (gonadal dysgenesis) and testicular oogenesis (hermaphroditism) in leopard frogs (*Rana pipiens*). Slower developing males even experienced oocyte growth (vitellogenesis). Furthermore, we observed gonadal dysgenesis and hermaphroditism in animals collected from atrazine-contaminated sites across the United States. These coordinated laboratory and field studies revealed the potential biological impact of atrazine contamination in the environment. Combined with reported similar effects in *Xenopus laevis,* the current data raise concern about the effects of atrazine on amphibians in general and the potential role of atrazine and other endocrine-disrupting pesticides in amphibian declines.** ***Key words:*** **amphibian, atrazine, endocrine disruption, hermaphrodite.** ***Environ Health Perspect*** **111:568–575 (2003). doi:10.1289/ehp.5932 available via *http://dx.doi.org/*** [Online 23 October 2002]

Atrazine is probably the most widely used herbicide in the world and one of the most common contaminants in ground and surface waters [U.S. Environmental Protection Agency (EPA) 1994]. Recently, Tevera-Mendoza et al. (2002) showed that atrazine exposure (21 ppb) for as little as 48 hr resulted in severe gonadal dysgenesis in African clawed frogs (*Xenopus laevis*). Further, we showed that atrazine induced hermaphroditism at concentrations of only 0.1 ppb (Hayes et al. 2002) when administered throughout larval development. Most water sources in the United States, including rainwater, can exceed the effective concentrations in these laboratory studies (Hayes et al. 2002). In addition, the concentration in our previous study (Hayes et al. 2002) is 30 times lower than the current drinking water standard (Hayes 1993). Despite the significance of the reported effects in *X. laevis*, both studies (Hayes et al. 2002; Tevera-Mendoza et al. 2002) were conducted in the laboratory on a single species; whether the effects of atrazine are widespread in amphibians and whether effects occur in the wild remained unanswered.

In the current study, we examined the effects of atrazine on leopard frogs (*Rana pipiens*), a U.S. native species, under controlled laboratory conditions. Once effects of atrazine were identified, we examined wild *R. pipiens* from a variety of habitats in areas with reportedly low atrazine use and areas with high atrazine use in a transect that extended from Utah to the Iowa–Illinois border. Further, we collected water samples and examined atrazine contaminant levels at each site. These coordinated laboratory and field analyses uniquely addressed the ecological significance and relevance of our initial laboratory studies.

## Materials and Methods

***Animal care for laboratory studies.*** Leopard frogs (*R. pipiens*) were obtained from Sensiba Marsh, Brown County, Wisconsin, and shipped overnight to the University of California at Berkeley. Eggs were allowed to hatch and then were apportioned into rearing tanks. Larvae (30/tank) were reared in 4 L aerated 10% Holtfreter's solution (Holtfreter 1931) and fed Purina rabbit chow (Purina Mills, St. Louis, MO). Food levels were adjusted as larvae grew to maximize growth. Experiments were carried out at 22–23°C with animals under a 12-hr light/12-hr dark cycle (lights on at 0600 hr).

***Larval laboratory exposures.*** Larvae were treated by immersion with nominal concentrations of 0, 0.1, or 25 ppb atrazine (98% pure; Chemservice, Chester, PA). Concentrations were confirmed by chemical analysis. Atrazine was predissolved in ethanol, and all treatments contained 0.0036% ethanol. Each treatment was replicated three times (30 larvae/replicate). Cages were cleaned, water changed, and treatments renewed every 3 days. All treatments were systematically relocated every 3 days to ensure that no treatments or tanks experienced position effects. Animals were exposed throughout the larval period from 2 days posthatching until complete tail reabsorption. In all experiments, all dosing and analyses were conducted blindly with color-coded tanks and treatments.

***General measurements.*** At metamorphosis (complete tail reabsorption), each animal was weighed and measured. Animals were euthanized in 0.2% benzocaine (Sigma Chemicals, St. Louis MO), assigned a unique identification number, fixed in Bouin's fixative, and preserved in 70% ethanol until further analysis.

***Histological analysis of gonads.*** All analyses were conducted blindly. Initially, the sex of all individuals was determined based on gross gonadal morphology using a Nikon SMZ 10A dissecting scope (Technical Instruments, Burlingame, CA). In the laboratory study, histological analysis was conducted on nine females per treatment and on all males. All histology was conducted according to Hayes (1995). In brief, tissues were dissected and dehydrated in graded alcohols followed by infiltration with Histoclear and paraffin (National Diagnostics, Atlanta, GA). Serial histological sections were cut at 8 µm through the entire gonad. Slides were stained in Mallory's trichrome stain and analyzed using a Nikon Optiphot 2 microscope (Technical Instruments). Images of gonads were recorded using a Sony DKC-5000 digital camera (Technical Instruments). For gonadal analysis, we examined every section from each gonad.

***Site selection for field studies.*** Initially, we chose study localities based on atrazine use, as determined by atrazine sales (Figure 1). All localities were between 39°N and 43°N latitude. Counties with < 0.4 kg/km$^2$ atrazine use were chosen as potential control sites, and areas with > 9.3 kg/km$^2$ atrazine use were chosen as potential atrazine-exposed sites. We began sampling in Utah on 15 July 2001 and moved eastward. In Utah, we chose one site (Juab County) in an area with < 0.4 kg/km$^2$ atrazine sales, and we collected in Cache County, Utah, with 0.4–2.4 kg/km$^2$ reported atrazine use. Carbon County, Wyoming, was considered a control site because the locality is not in the vicinity of farms, and the county (most of the

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state, in fact) reports < 0.4 kg/km$^2$ atrazine sales. In Nebraska, we chose one site in York County with high atrazine use, and one site in Cherry County where atrazine sales were < 0.4 kg/km$^2$. All sites in Iowa were considered exposed sites, except a single site in a wildlife protection area in Iowa. We stopped sampling at the Iowa–Illinois border because *R. pipiens* populations are reportedly low or threatened in Illinois and Indiana.

***Frog and water sampling from field localities.*** At each site (Figure 1), we collected 100 animals (eight sites, for a total of 800 animals). We selected small individuals in an attempt to sample newly metamorphosed animals. Immediately after collection, animals were euthanized in benzocaine, fixed in Bouin's fixative for 48 hr, and preserved in 70% ethanol. Animals were returned to the laboratory and measured, the sex of each was determined, and histological analysis was conducted on the gonads of 20 males from each site and a subset of females from each site. Histology was conducted as described for laboratory studies.

***Chemical analysis.*** At each site, we collected water (100 mL) in clean chemical-free glass jars (Fisher Scientific Co, Houston, TX) for chemical analysis. Water samples were frozen on dry ice immediately upon collection and maintained frozen (–20°C) until analysis. Atrazine levels from all sites were determined by PTRL West Inc. (Hercules, CA). Water samples were extracted in organic solvent, followed by aqueous/organic extraction. Samples were analyzed by liquid chromatography/mass spectrophotometry using the daughter ion. Duplicate samples were analyzed at the Hygienic Laboratory (University of Iowa, Iowa City, Iowa). The Hygienic Laboratory used U.S. EPA method 507 (U.S. EPA 1988). In brief, water samples were extracted in organic solvent and subjected to gas chromatography with a nitrogen phosphorous detector. Both analytical laboratories received coded samples and were not aware of the collection localities. In addition, each laboratory received negative controls that contained only Holtfreter's solution (Holtfreter 1931) and positive controls that contained mixtures of pesticides at both 0.1 and 10 ppb. Detection limits were 0.1 ppb for both laboratories, and data for duplicated samples were accepted only when both laboratories reported results within 10% of each other. Reported values reflect the higher estimate rounded to the nearest 0.1. In addition to atrazine, PTRL West Inc. reported the atrazine metabolites diaminochlorotriazine (DAC), deisopropylatrazine (DIA), and deethylatrazine (DEA), as well as triazines (simazine and hexazinone) and two other herbicides (diuron and norflurazon) from all sites. In addition to atrazine, the following pesticides were reportedly used at site 5 in Nebraska: herbicides (metolachlor, alachlor, glyphosate), fungicides (metalaxyl, nicosulfuron, propiconazole), and insecticides (β-cyfluthrin, λ-cyhalothrin, tebupirimphos). These pesticides were analyzed using appropriate methods by the Iowa Hygienic laboratories for site 1 (Utah), site 3 (Wyoming), and site 5 (Nebraska) only.

## Results

***Gonadal analysis in laboratory-reared animals.*** Control animals were sexually differentiated at metamorphosis. The earliest males to metamorphose had solid testicular lobules. Primary spermatogonia were recognized in the lobules of some animals (Figure 2). Animals that metamorphosed later had open lobules that contained both primary spermatogonia and spermatids. Females had numerous oocytes in their gonads and a central ovarian cavity (Figure 3).

Atrazine-treated males (0.1 and 25 ppb) were sexually differentiated at metamorphosis as well, but 36 and 12% of the males treated with 0.1 and 25 ppb atrazine, respectively, suffered from gonadal dysgenesis—underdeveloped testes with poorly structured, closed lobules (or no lobules at all) and low to absent germ cells (Figure 4). Further, 29% of the 0.1

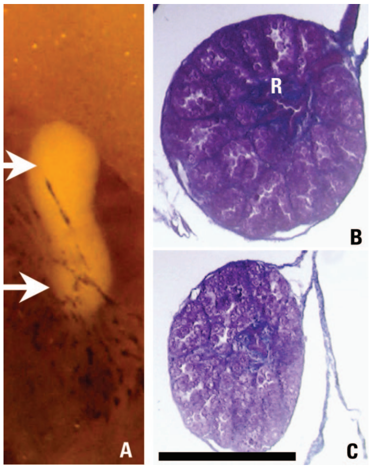


**Figure 2.** Right testis of a control male leopard frog (*R. pipiens*) at metamorphosis. (*A*) The gonad is still attached to the kidney. White arrows show the position of transverse cross-sections shown in (*B*), the anterior section, and (*C*), the posterior section. Testicular lobules were well developed and the specimen had both primary spermatogonia and spermatids present. In general, testes were more differentiated (contained more distinct tubules and germ cells) anteriorly than posteriorly. R = rete testis. See "Materials and Methods" for details of histological analysis. Bar = 1.0 mm for (*A*) and (*B*) and 250 µm for (*C*).

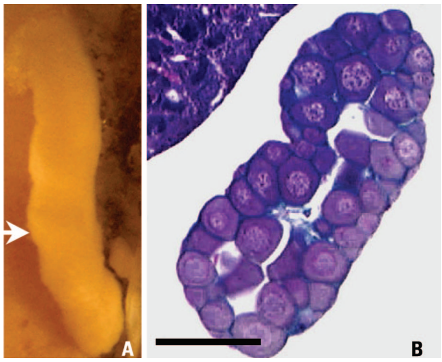


**Figure 3.** Right ovary from a control female. (*A*) The gonad is still attached to the kidney. The white arrow shows the position from which the transverse cross-section shown in (*B*) was taken. The ovary contains a large number of oocytes, and the ovarian cavity is visible. See "Materials and Methods" for details of histological analysis. Bar = 1.0 mm for (*A*) and 250 µm for (*B*).

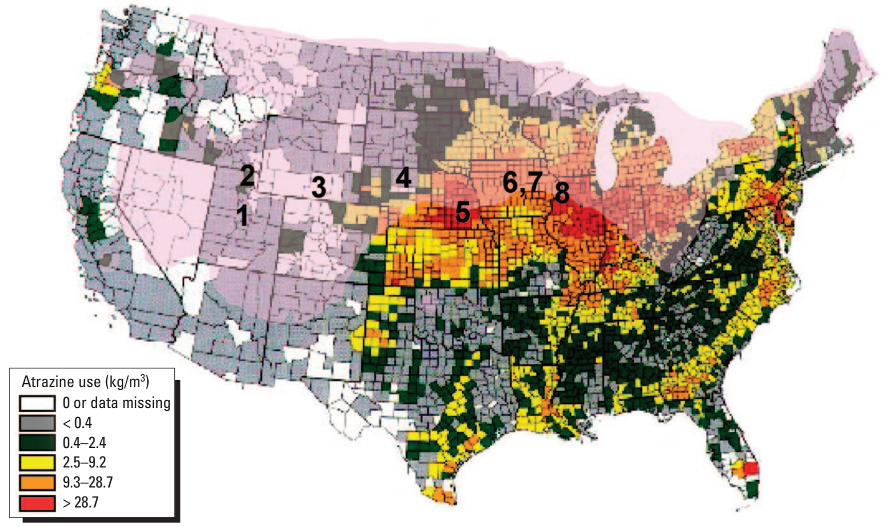


**Figure 1.** Map of the United States showing atrazine use based on sales (Battaglin and Goolsby 1995). The pink overlay shows the natural range for leopard frogs (*R. pipiens*) in the United States. Numbers indicate sites where water (for chemical analysis) and frogs (for histological analysis of gonads) were collected. Collection sites are numbered and correspond to site numbers used in Table 1. Reprinted from Hayes et al. (2002) with permission from *Nature*.

ppb-treated animals and 8% of the animals treated with 25 ppb displayed varying degrees of sex reversal. The testicular lobules of sex-reversed males contained oocytes when observed histologically (Figure 5), and males that metamorphosed later contained large numbers of oocytes (Figures 6 and 7). Males that appeared to undergo complete sex reversal had gonads almost completely filled with oocytes and only a limited number of lobules remained (Figure 7). In two males, oocytes were vitellogenic and protruded through the testicular lobules, which made the oocytes observable upon gross analysis of the gonads (Figure 8). Control males never contained testicular oocytes, although two control males contained two to three degenerating extragonadal oocytes (not within lobules), and a single control male showed gonadal dysgenesis (Figure 9). There were no observable effects in atrazine-treated females.

***Field localities.*** Once effects were identified in laboratory-reared animals, we conducted a study of gonadal morphology in field-collected *R. pipiens* to determine if animals exposed in the wild displayed similar abnormalities. Localities for collections are

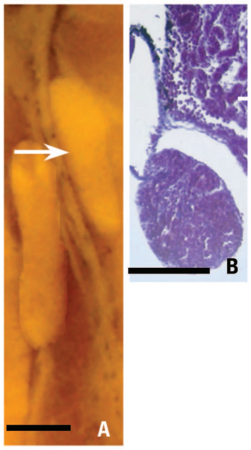
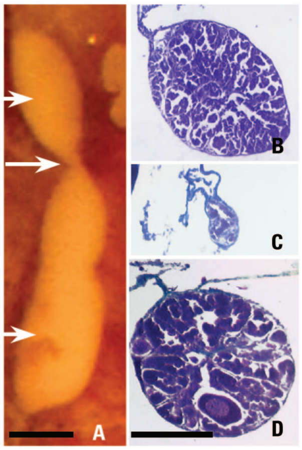


**Figure 4.** Gonadal dysgenesis in testis from a male *R. pipiens* treated with 0.1 ppb atrazine. (*A*) Testis fixed in Bouin's solution showing poor lobular development and gonads that are devoid of germ cells; bar = 250 µm. (*B*) Transverse cross-section taken from the area indicated by the arrow in (*A*); bar = 100 µm. See "Materials and Methods" for details of histological analysis.



**Figures 5.** Lobed gonad from a male *R. pipiens* treated with 0.1 ppb atrazine. (*A*) Gonad fixed in Bouin's solution. Arrows show areas where transverse cross-sections were taken; bar = 250 µm. (*B*) Most anterior section; the testicular lobules are open and distinct and contain spermatids. (*C*) Center section, containing three lobules. (*D*) Most posterior section; well-developed lobules contain mostly spermatids, but one lobule is devoid of spermatids or spermatogonia and contains single large oocytes each. A single oocyte is seen in (*D*). Bar = 100 µm for (*B*–*D*). See "Materials and Methods" for details of histological analysis.

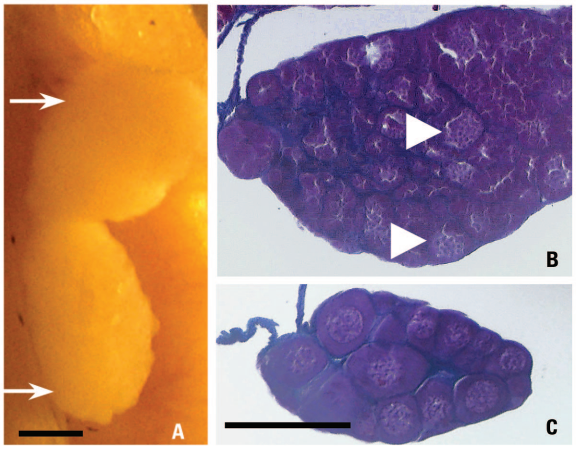


**Figure 6.** Right lobed gonad of a treated male *R. pipiens* (0.1 ppb atrazine) with anterior testes and developing posterior ovary. (*A*) Gonad fixed in Bouin's solution; white arrows show areas where transverse cross-sections were taken. (*B*) Anterior portion is testicular, with lobules that contain spermatids; white arrows indicate lobules with spermatids. (*C*) Posterior portion of the gonad has large testicular oocytes. Bar = 250 µm for (*A*–*C*). See "Materials and Methods" for details of histological analysis.

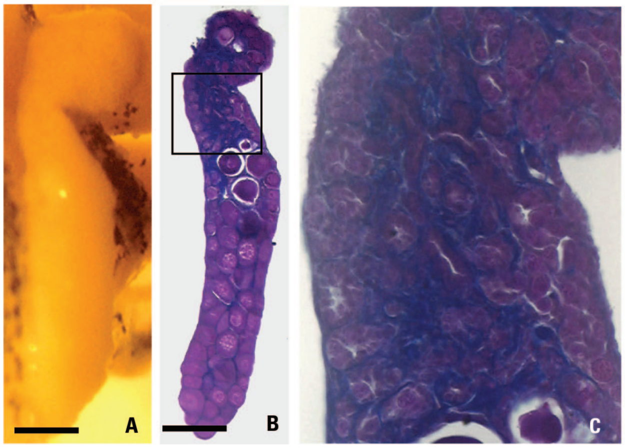


**Figure 7.** Left gonad of a treated male *R. pipiens* (0.1 ppb atrazine) with anterior testes and developing posterior ovary (same animal shown in Figure 6). (*A*) Bouin's-fixed section; bar = 250 µm. (*B*) Sagittal section of the left gonad, which is almost completely converted into an ovary. The entire posterior gonad is ovarian, and oocytes are beginning to grow in the anterior portion; bar = 250 µm. (*C*) Magnification of a small portion of the gonad [outlined by the box in (*B*)] still contains testicular lobules, but the lobules lack germ cells; bar = 55.5 µm. See "Materials and Methods" for details of histological analysis.

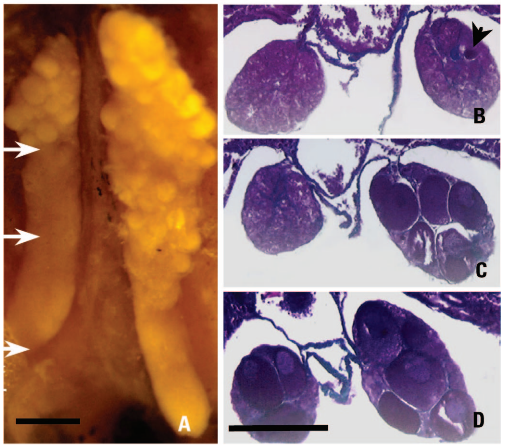


**Figure 8.** Gonads from a treated male *R. pipiens* (0.1 ppb atrazine) with vitellogenic testicular oocytes. (*A*) Bouin's-fixed section; bar = 250 µm. The posterior portion of the gonad is filled with oocytes that are protruding through the testicular lobules and can be seen on the surface of the gonad; white arrows show areas where transverse cross-sections were taken. (*B*) Transverse cross-sections showing that the anterior testis has poorly developed testicular lobules; the black arrowhead shows a tangentially sectioned oocyte. (*C*) and (*D*) Large vitellogenic oocytes in the posterior portion of the gonads. Bar = 250 µm for panels (*B*–*D*). See "Materials and Methods" for details of histological analysis.

shown in Figure 1. We chose four sites from potential control/uncontaminated areas (counties in Utah, Wisconsin, and Nebraska that reported atrazine sales < 0.4 kg/km$^2$ and a nonagricultural site in Iowa) and four contaminated areas (Cache County, Utah, the single county that reported > 0.4 kg/km$^2$ in atrazine sales, one site in an agricultural area in Nebraska, and two similar sites in Iowa). In addition to varying in the amount of atrazine use, the habitats at collecting sites ranged from wildlife protection areas to agricultural runoff and cornfields (Figure 10, Table 1). Chemical analysis of water samples collected from each site revealed that none of the sites were atrazine free, and only one site (Juab County, Utah) had atrazine levels < 0.2 ppb (Table 1). Sites in Utah and Wyoming did not have detectable levels of atrazine metabolites (Figure 11A). None of the other pesticides assayed were present at any site except metolachlor, which was present at site 5 (York, NE) at 0.39 ppb.

***Analysis of gonads from field-collected animals.*** Testicular oocytes were identified in males from seven of eight sites (Table 1, Figure 11B). All sites associated with atrazine sales that exceeded 0.4 kg/km$^2$ and atrazine contaminant levels that exceeded 0.2 ppb had males that displayed sex reversal similar to those abnormalities induced by atrazine in the laboratory (Figures 12–15). In addition, in high-use York County, Nebraska, 28% of the males examined had gonadal dysgenesis (Figure 13), and testicular oocytes were found in a single male. The poorly developed testicular lobules that lacked germ cells observed in males from the corn field in York County resembled gonadal dysgenesis observed in males exposed to 0.1 ppb atrazine in our laboratory study and effects described in *X. laevis* (Tevera-Mendoza et al. 2002). Site 3, on the North Platte River in Wyoming, was not associated with direct agricultural runoff, but had the highest incidence and the most advanced cases of hermaphroditism. Most of the males observed from this site (92%) had testicular oocytes, and many animals showed advanced stages of complete sex reversal. All other sites had varying frequencies and severities of gonadal abnormalities. There were no observable abnormalities in females from any of the localities.

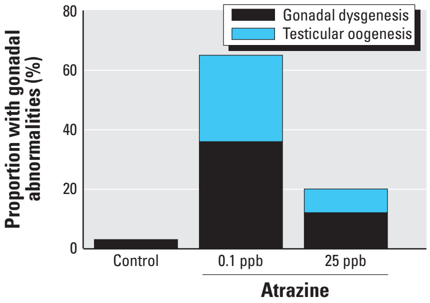


**Figure 9.** Frequency (percent) of gonadal abnormalities in males treated with atrazine in the laboratory.

## Discussion

Atrazine exposure disrupted gonadal development in exposed larvae. Testicular tubules were poorly developed in exposed animals (gonadal dysgenesis), germ cells appeared reduced, and oocytes were allowed to develop (testicular oogenesis) in animals identified as hermaphrodites. In at least two animals, oocytes were vitellogenic and protruded through the testes. Furthermore, effects were more pronounced at the lower dose (0.1 ppb). Widespread atrazine contamination was accompanied by observations of hermaphroditic animals in the field. Combined, these studies suggest that atrazine impacts amphibians in the wild.

***Relevance to previous work.*** In a previous study, Allran and Karasov (2000) examined the effects of atrazine on *R. pipiens*, but they used much higher doses and did not examine gonadal differentiation. Thus, their study did not identify the abnormalities that we observed here. The current study supports our previous findings of atrazine-induced hermaphroditism in *X. laevis* (Hayes et al. 2002) as well as the findings of Tevera-Mendoza et al. (2002), who showed retarded gonadogenesis and decreased germ cell numbers in atrazine-exposed males. Males with testicular oogenesis had lobed gonads similar to gonads of some atrazine-exposed males described in our studies of

**Figure 10**. Habitats at collection localities where animals and water were collected for analysis. (*A*) Site 1, Juab County, Utah; (*B*) site 2, Cache County, Utah; (*C*) site 3, Carbon County, Wyoming; (*D*) site 4, Cherry County, Nebraska; (*E*) site 5, York County, Nebraska; (*F*) site 6, Polk County, Iowa; (*G*) site 7, Polk County, Iowa; and (*H*) site 8, Clinton County, Iowa. Detailed coordinates and descriptions of habitats are given in Table 1.

*X. laevis*. Furthermore, atrazine exposure resulted in testicular oogenesis and even induced growth (vitellogenesis) of the oocytes in slower developing males, but had no effect in females. Atrazine does not bind the estrogen receptor (Tennant et al. 1994), but studies in reptiles (Crain et al. 1997), mammals (Sanderson et al. 2000, 2001), and fish (Sanderson et al. 2001) showed that atrazine induces aromatase and thereby increases the production of endogenous estrogen. The demasculinization (failure to induce spermatogenesis) and feminization (induction and growth of oocytes) observed in the current study and previous work (Hayes et al. 2002; Tevera-Mendoza et al. 2002) are explainable via the proposed mechanism.

***Effects on germ cell differentiation.*** Witschi (1929) suggested that some species/populations of *Rana* display rudimentary hermaphroditism in which undifferentiated races of Ranid frogs had ovaries with eggs anteriorly and testicular nodules (which did not contain oocytes) posteriorly. This developmental pattern was not observed in the population of *R. pipiens* used in our current study. Even atrazine-treated animals did not display the morphology described by Witschi (1929). In atrazine-exposed males, testicular oocytes were always in the posterior section of the gonads. In addition, testicular oogenesis and hermaphroditism were never observed in control animals in the current study or in other unpublished observations of *R. pipiens* in our laboratory, including more than 7,000 individuals and four populations (Utah, Nebraska, Wisconsin, and Connecticut).

Three control males had up to three extragonadal degenerating oocytes (never within the testicular lobules) at the posterior end of the gonads, however. Normally, germ cells migrate into the developing gonad from the yolk or gut endoderm (depending on the species). Primordial germ cells that fail to enter the developing testes become oocytes by default, even in mammals (McClaren 1995; Nakatsuji and Chuma 2001), but eventually degenerate. The current data suggest that atrazine demasculinized the gonads of exposed males. Instead of releasing the putative spermatogenesis-inducing factor (Witschi 1957), which would result in the degeneration of oocytes, atrazine-exposed males supported differentiation and even growth of these oocytes.

***Low-dose effects.*** The observation that 0.1 ppb atrazine was more effective than the higher dose (25 ppb) is interesting. Both the proportion of males with gonadal dysgenesis and the proportion with testicular oocytes (hermaphrodites) were higher at the lower dose. Low-dose effects have been described for a number of endocrine disruptors (estrogenic compounds and antiandrogenic compounds), and some compounds even produce different effects at different doses and in different tissues (Akingbemi and Hardy 2001). Low-dose effects of demasculinizing and feminizing environmental endocrine disruptors on male development have been of particular concern (Akingbemi and Hardy 2001). Furthermore, similar perplexing effects are known for 17β-estradiol on sex differentiation in *R. pipiens* (Richards and Nace 1978). Low doses of estradiol (< 0.07 μM) do not affect sex differentiation, higher doses (0.07–0.18 μM) produce 100% females, and still higher doses (> 3.69 μM) produce 100% males (Hayes 1998). A mixture of normal males, females, and intersexes are produced at doses between 0.18 and 3.69 μM.

***Relevance to wild amphibian populations.*** The use of a U.S. native species in the current study allowed us to assess the realized impact of atrazine on wild amphibians. Wild populations that contained males with gonadal dysgenesis and testicular oocytes (hermaphrodites) were associated with localities with atrazine use and/or atrazine contamination. Reeder et al. (1998) described testicular oocytes in field-collected frogs (*Acris creptians*) and suggested that atrazine may be involved in this abnormality, but did not have laboratory data to support the suggestion. Atrazine may not be the only compound that induces testicular oogenesis, however. There may be many chemicals, natural products, and even populations that naturally display this phenomenon (Witschi 1929). Nevertheless, the present study showed that atrazine induced testicular oogenesis and hermaphroditism in a population that does not show this developmental pattern under controlled laboratory conditions, and that hermaphroditism in wild *R. pipiens* is associated with atrazine use and contamination.

***Extent of atrazine contamination.*** The present study demonstrated the extent of atrazine contamination and its potential impact. The locality in Wyoming (North Platte River) with the highest frequency of sex reversal (92% of the males) is not in the vicinity of farms, nor is it in a county that reports significant atrazine use. The North Platte River originates in Grand County, Colorado, which has some corn production and atrazine use. Atrazine contamination in other parts of the Platte River system is well documented (Kimbrough and Litke 1995). Thus, amphibians at the locality in Wyoming that does not report significant atrazine use may be at risk

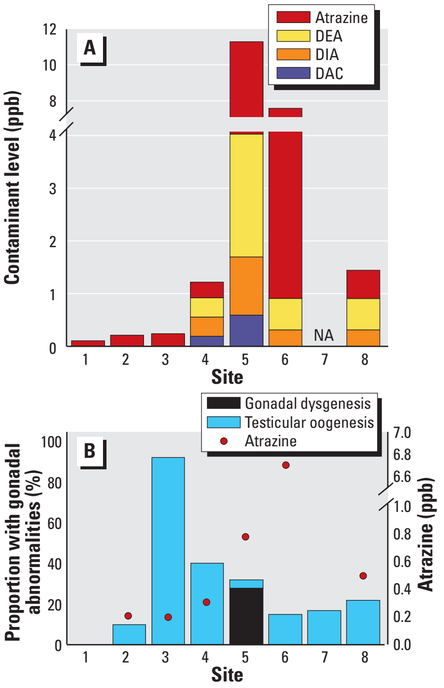


**Figure 11.** Presence of atrazine and metabolites: atrazine, DIA, DEA, and DAC at sites 1–8 (*A*) and frequency (%) of gonadal abnormalities in males collected from the wild (*B*). Localities and descriptions of each site are listed in Table 1. Atrazine contaminant levels for each site are also indicated in (*B*). Atrazine was not detected at site 1, and data were not available for site 7. (*B*) is modified from Hayes et al. (2002) with permission from *Nature*.

**Table 1.** Dates, localities, descriptions, and atrazine levels for field localities.

| Site | Date | State | County | Latitude | Longitude | Altitude (m) | Source | Habitat | Atrazine (ppb) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 15 July 2001 | Utah | Juab | 111°52.23W | 39°46.63N | 1,500 | Pond | Graze land | ND |
| 2 | 17 July 2001 | Utah | Cache | 111°50.14W | 39°43.40N | 1,555 | Pond | Golf course | 0.2 |
| 3 | 19 July 2001 | Wyoming | Carbon | 107°03.28W | 41°51.68N | 1,952 | River | Wildlife area | 0.2 |
| 4 | 23 July 2001 | Nebraska | Cherry | 101°42.89W | 42°41.67N | 1,031 | Pond | Prairie | 0.3 |
| 5 | 22 July 2001 | Nebraska | York | 97°22.38W | 40°55.88N | 480 | Ditch | Corn field | 0.8 |
| 6 | 26 July 2001 | Iowa | Polk | 93°27.39W | 41°48.11N | 252 | Ditch | Corn field | 6.7 |
| 7 | 28 July 2001 | Iowa | Polk | 93°25.50W | 41°47.47N | 246 | Marsh | Wildlife area | NA |
| 8 | 28 July 2001 | Iowa | Clinton | 90°21.28W | 41°44.46N | 211 | Stream | River valley | 0.5 |

Abbreviations: NA, not available; ND, not detectable. Levels reported by the two analytical laboratories were inconsistent (not within 10%); one laboratory (PTRL West) reported nondetectable levels.

from contamination from Colorado. Similarly, atrazine contamination of ground and well water has been reported in Utah, even in areas where atrazine is not used heavily (Thiros 2000). Contamination of groundwater in Utah, transport of atrazine to Wyoming via the North Platte River, the presence of atrazine in Cherry County, Nebraska (where no atrazine use is reported), and findings of hermaphrodites at these localities further demonstrate the problem of widespread atrazine contamination.

Additional water collections and contaminant analysis revealed the difficulties in determining the contaminant levels that larvae experience. Even though atrazine reportedly has a short half-life (as little as 8 days) (Solomon et al. 1996), we measured atrazine contamination (> 0.2 ppb) in irrigation ditches in York County, Nebraska, even on 31 March 2001. According to on-site pesticide application records at the time of water collections, atrazine had not been applied since 19 May 2000. Thus, atrazine levels in this area never decreased below the determined biologically active levels. Furthermore, atrazine levels varied from 15.2 ppb to 0.8 ppb over a 24-hr period (22–23 July 2001, respectively) as a result of evaporation followed by an increase in the water level from irrigation and runoff. In addition, even though atrazine is applied directly only twice per year at this locality, the water source used for irrigation had atrazine levels of 0.7 ppb, so levels of atrazine capable of inducing testicular oogenesis are continuously applied to these fields. Runoff from cornfields in this area also flowed into adjacent organic farms and wildlife protection areas, resulting in atrazine contamination in excess of 15 ppb at both the organic farm and in the refuge on 23 July 2001, even though atrazine had not been applied since 13 May 2001 at this locality. Further, on 23 July 2001, atrazine levels in rainwater and tap water in York County, Nebraska, were 0.4 ppb and 0.3 ppb, respectively. Thus, even rain and tap water in York County contained enough atrazine to disrupt normal male development in amphibians. Finally, as suggested in Figure 16, atrazine levels were likely at their lowest at the time of our collections in July, and the levels likely peak during critical stages of larval development.

***Difficulties with quantitative analyses and predictive capabilities.*** Although we attempted to predict localities where hermaphrodites might occur based on atrazine use, the movement of atrazine into areas such as Carbon County, Wyoming, via the North Platte River, the presence of atrazine in Cherry County, Nebraska (where atrazine use is not reported), and local use of atrazine in areas that do not report high use make such predictions difficult. In addition, habitat type and local land-use history are not good predictors because of the transport of atrazine (e.g., the hermaphrodites identified in the wildlife area

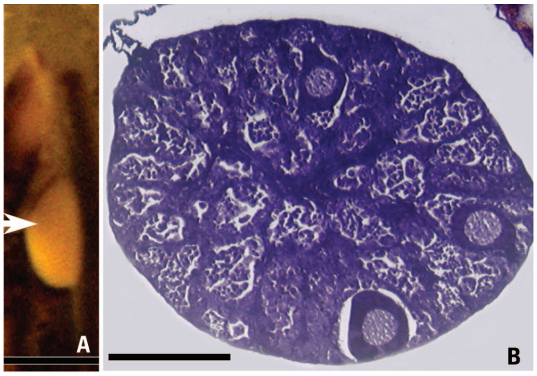


**Figure 12.** Gonad of a male *R. pipiens* from site 8 (Clinton, Iowa). (*A*) Left testis fixed in Bouin's solution. The white arrow shows the area where the transverse cross-section was taken; bar = 0.1 mm. (*B*) Transverse cross-section taken from the area indicated by the white arrow in (*A*). Well-developed testicular lobules with spermatids, and three lobules that contain both spermatids and a single large oocyte each; bar = 250 µm. See "Materials and Methods" for details of histological analysis.

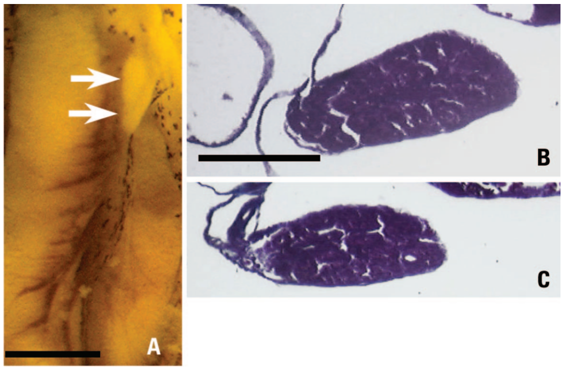


**Figure 13.** Gonad of a male *R. pipiens* from site 5 (York County, Nebraska). This animal displayed gonadal dysgenesis as seen in 28% of the animals from that locality. (*A*) Left testis fixed in Bouin's solution. White arrows show the areas where the transverse cross-sections were taken; bar = 0.1 mm. (*B*) Anterior and (*C*) posterior cross-sections; bar = 250 µm. This morphology was similar to that displayed by 36% of the animals exposed to 0.1 ppb atrazine in the laboratory. See "Materials and Methods" for details of histological analysis.

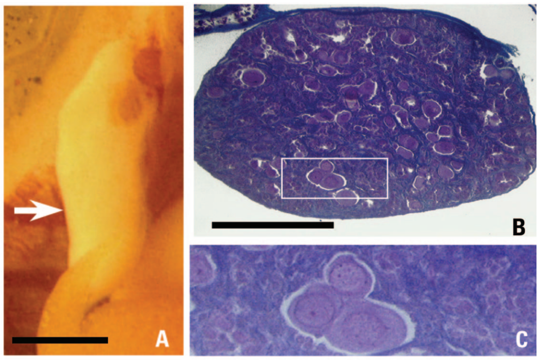


**Figure 14.** Gonad of a male *R. pipiens* from Carbon County, Wyoming. (*A*) Gonad fixed in Bouin's solution; bar = 0.1 mm. (*B*) Transverse cross-section taken from the area indicated by the white arrow in (*A*); numerous testicular oocytes fill 100% of the testicular lobules. (*C*) Magnified view of the boxed area in (*B*) showing some lobules with as many as three oocytes; bar = 500 µm for (*B*) and (*C*). See "Materials and Methods" for details of histological analysis. (*B*) Reprinted from Hayes et al. (2002) with permission from *Nature*.

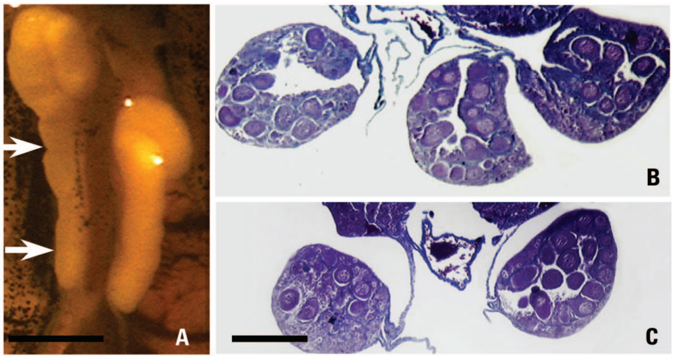


**Figure 15.** Gonads of a *R. pipiens* hermaphrodite from Carbon County, Wyoming, undergoing what appears to be complete sex reversal. (*A*) Gonads (fixed in Bouin's solution) are becoming convoluted similar to an ovary. White arrows show the areas where the transverse cross-sections were taken; bar = 0.1 mm. (*B*) Anterior and (*C*) posterior transverse cross-sections reveal that the gonads contain numerous oocytes; bar = 250 µm. The animal has developed an ovarian cavity and lost its lobular structure. See "Materials and Methods" for details of histological analysis.

in Iowa, Cherry County, Nebraska, and Carbon County, Wyoming).

Further, low-dose effects and the extent and variability in atrazine contamination make quantitative analyses difficult. Because 0.1 ppb was effective in the laboratory (and in fact more effective than 25 ppb), we did not identify a concentration that is below threshold in our laboratory studies. In *X. laevis* (Hayes et al. 2002), 0.01 ppb was ineffective, but hermaphroditism was observed at 0.1–200 ppb. Even if a minimum concentration were identified, both the current and the previous study (Hayes et al. 2002) suggest that there is not a linear dose response. In fact, both studies imply that there is an "inverted U" (parabolic) response (Chen 2001) in which very low concentrations may be without effect, higher concentrations have decreasing effectiveness, and intermediate low concentrations are most effective. There does not appear to be a relationship between atrazine concentrations and the number or size of testicular oocytes, but longer exposure times may be associated with increasing numbers of testicular oocytes, size of testicular oocytes, and the extent of testicular conversion to ovaries.

Even if the dose–response pattern were understood, variation in atrazine levels from locality to locality and even from day to day at a single locality make it difficult to predict where high frequencies of affected males might occur. In addition such statistical models would involve nonparametric statistics (such as G-tests) that rely on testing observed frequencies of hermaphrodites against predicted frequencies. Either expected frequencies would be set to zero, or we would assume some natural expected frequency of hermaphroditism that may vary between populations. It is difficult to determine the predicted (natural) frequencies, although we have reared animals (more than 7,000) from Utah, Nebraska, Wisconsin, and Connecticut in the laboratory and never observed testicular oogenesis or hermaphroditism unless animals were exposed to atrazine. Despite difficulties that limit quantitative analyses at this time, testicular oogenesis and hermaphroditism always occurred at localities associated with atrazine exposure and were absent only at the single site with < 0.2 ppb atrazine contamination (Juab County, Utah).

***The threat to amphibians.*** Findings of similar effects of atrazine on sexual development in two diverse species (*X. laevis* and *R. pipiens*), show that effects of atrazine are not restricted to a single species, and in fact likely present a problem for amphibians in general. The pattern of atrazine use creates even more concern. As shown in Figure 16, atrazine levels are highest during larval development (Conant 1998; Stebbins 1985) for most local species. Applied as a preemergent, atrazine contamination of water sources peaks with spring rains. The timing of atrazine contamination of water sources directly coincides with amphibian breeding activities, as many amphibians reproduce during early spring rains. Given the identified effects of atrazine in the laboratory, combined with the apparent correlation of atrazine contamination with similar morphologies in the wild and the pattern of atrazine use, the potential impact of atrazine on amphibians is significant.

Many amphibian species are in decline (Blaustein and Kiesecker 2002; Gardner 2001; Wake 1991), and *R. pipiens* populations are declining in many locations in Indiana and Illinois. Juvenile *R. pipiens* were abundant at all of our collection sites, however, including agricultural areas in Iowa and Nebraska. The abundance of frogs at these sites suggests that the effects are reversible, that some percentage of the population does not show this response, that these developmental abnormalities do not impair reproductive function at sexual maturity, and/or that continuously exposed populations have evolved resistance to atrazine. In fact, although gonadal dysgenesis may be induced by atrazine (based on our laboratory studies), it may be a mechanism of resistance as well. If lobular formation and germ cell differentiation are delayed until after metamorphosis, then portions of the population that display gonadal dysgenesis may escape atrazine-induced sex reversal because they would undergo sex differentiation after metamorphosis once they leave the contaminated water. This hypothesis is testable in the laboratory, as the proportion of exposed males with gonadal dysgenesis at metamorphosis should reflect the proportion of normal males in the population after metamorphosis. In addition, higher proportions of affected males at a locality that only periodically experiences high contaminant levels (such as Wyoming) may reflect that these populations have not been under the same intensity of selection for atrazine resistance. Periodic fluctuations in atrazine contamination may affect large proportions of some populations from year to year, but unexposed animals, or animals from previous years, may continue to breed. Further studies are needed to address these questions and the realized impact of atrazine on exposed populations.

There are likely many factors involved in amphibian declines. Endocrine disruption by

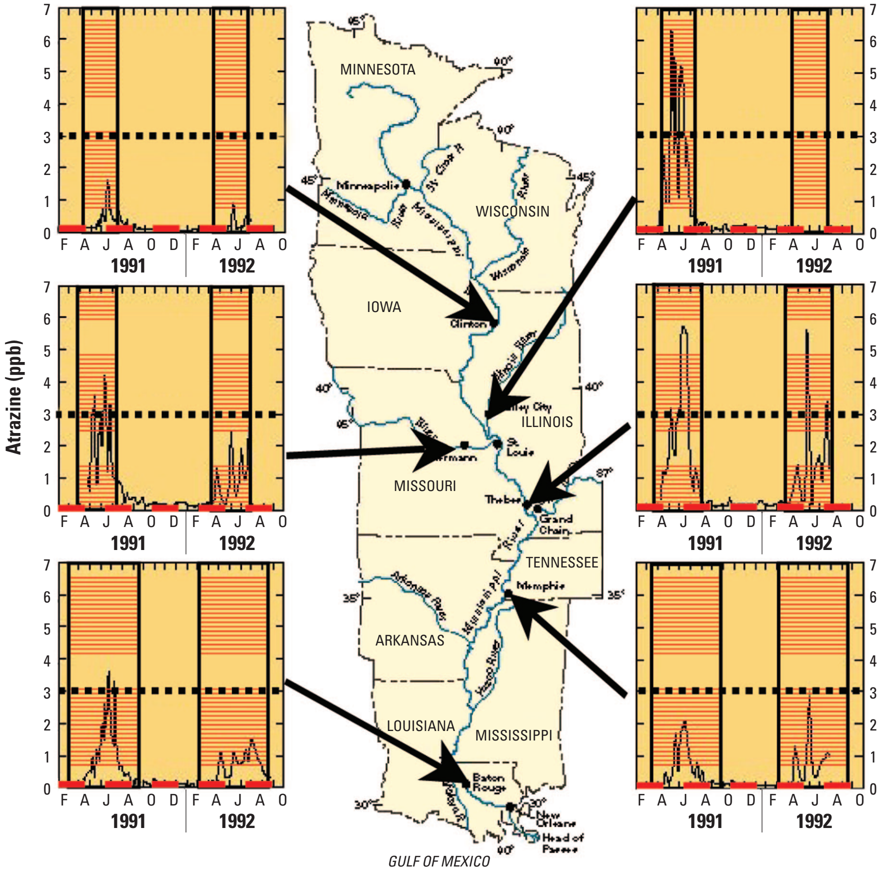


**Figure 16.** Atrazine contamination along the Mississippi River from 1991 to 1992 (Goolsby and Pereira 1995). The horizontal axis shows months (February, April, June, August, October, and December) over 2 years. The dashed horizontal line shows the maximum contaminant level set by the U.S. EPA (3 ppb), and the red horizontal line shows the dose effective at producing hermaphrodites in the laboratory (0.1 ppb). The vertical red bars for each year show the timing of larval development for amphibians in each region.

pesticides is but one potential cause, and atrazine only one such compound. However, given the widespread use and ubiquitous contamination by atrazine, its pattern of use, and its potency as an endocrine disruptor, atrazine likely has a significant impact on amphibian populations. In particular, given recent evidence that atrazine potentiates parasitic infections in amphibians (Kiesecker 2002) in addition to its impact on reproductive development, the role of atrazine in amphibian declines is of particular concern. Further, enhancement of atrazine effects when mixed with other pesticides, as indicated in our ongoing studies, must be explored.

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