Low Levels of the Herbicide Atrazine Alter Sex Ratios and Reduce Metamorphic Success in *Rana pipiens* Tadpoles Raised in Outdoor Mesocosms

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**Background:** There are conflicting reports regarding the effects of atrazine (ATZ) on amphibian development. Therefore, further studies are needed to examine the potential mechanisms of action of ATZ in amphibians.

**Objectives:** Our aim in this study was to determine whether low concentrations of ATZ affect gonadal development and metamorphosis in the Northern leopard frog, *Rana pipiens*.

**Methods:** Tadpoles were exposed in outdoor mesocosms to nominal concentrations of 0.1 and 1.8 µg/L of formulated ATZ from Gosner stage 27 (G27) to metamorphic climax (G42). Exposure to 17α-ethinylestradiol (EE2; 1.5 µg/L) provided a positive control for induction of testicular oocytes in males. Endocrine-related gene expression and gonadal histopathology were examined at G42 and in a subset of metamorphic G34 tadpoles that failed to metamorphose.

**Results:** Gonadal gross morphology revealed that the 1.8-µg/L ATZ treatment produced 20% more females compared with the control. Histologic analysis revealed that 22% of EE2-treated males had testicular oocytes, whereas none were observed in any animals from the control or either ATZ groups. ATZ increased brain estrogen receptor α mRNA to 2.5 times that of the control at metamorphosis and altered liver levels of 5β-reductase activity at metamorphosis. In contrast, brain aromatase mRNA level and activity did not change. ATZ treatments significantly reduced metamorphic success (number of animals reaching metamorphosis) without affecting body weight, snout–vent length, or age at metamorphosis. Gene expression analysis indicated that ATZ decreased the expression of deiodinase type 3 in the tail at metamorphosis.

**Conclusions:** Our study indicates that exposure to low concentrations of ATZ in experimental mesocosms alters gonadal differentiation and metamorphosis in developing *R. pipiens*.

**Key Words:** 5β-reductase, amphibians, aromatase, atrazine, estrogen activity, feminization, gonadal development, metamorphosis, Northern leopard frog, real-time RT-PCR. Environ Health Perspect 118:552–557 (2010). doi:10.1289/ehp.0901418 [Online 19 November 2009]

There is controversial evidence that the widely used herbicide atrazine (ATZ) may alter gonadal development by affecting gonadal steroidogenesis through alteration of aromatase activity (Hayes et al. 2002b). Aromatase (cyp19) is a cytochrome P450 enzyme that converts testosterone into estradiol (Lephart 1996) and androstenedione into estrone (Simpson et al. 1994). In numerous fish, reptile, and amphibian species, cyp19 induction or inhibition produces female-biased or male-biased sex ratios, respectively (Charbard and Dournon 1999; Navarro-Martin et al. 2009; Richard-Mercier et al. 1995). Induction of *in vitro* cyp19 activity has been reported in human cell lines after exposure to ATZ (Heneveur et al. 2004; Holloway et al. 2008). However, several other studies have not observed such responses in amphibians (Coady et al. 2005; Hecker et al. 2005a; 2005b; Oka et al. 2008). The underlying reasons for these differences and the mechanism through which ATZ may disrupt vertebrate development remain unclear.

In the present study, we investigated alternative mechanisms through which ATZ may induce estrogen-like effects in amphibians. These mechanisms include the induction of estrogen receptor α (*eralpha*), which is activated upon estrogen binding and has been recognized as an estrogenic biomarker of estrogenic exposure (Lutz et al. 2005). Studies have shown that after treatment with estrogenic substances, *eralpha* expression increased in *Rana pipiens* tadpole brain [17α-ethinylestradiol (EE2); Duarte et al. 2006], the whole body of * Xenopus laevis* tadpoles (bisphenol A; Levy et al. 2004), and fish liver (EE2; Filby et al. 2007). The 5β-reductase (sr5beta) pathway is also potentially involved in the feminization of developing amphibians (Duarte-Guterman et al. 2010). A member of the aldol-keto reductase superfamily, sr5beta can regulate androgen bioavailability by catalyzing the conversion of testosterone to 5β-dihydrotestosterone (5β-DHT) reviewed by Langlois et al. (2009). Therefore, we hypothesized that exposure to ATZ alters *eralpha* mRNA level and sr5beta activity in the target tissues of exposed tadpoles.

There is also controversial evidence that ATZ affects amphibian development and metamorphosis (Coady et al. 2004; Freeman and Rayburn 2005). Several studies have reported developmental defects in amphibians after ATZ exposure (Brodeur et al. 2009; Coady et al. 2004; Freeman et al. 1998; Lenkowski et al. 2008; Storrs and Kiesecker 2004). However, several other studies have not found any evidence that ATZ disrupts amphibian development even in the same species (Carr et al. 2003; Coady et al. 2005; Diana et al. 2000; Oka et al. 2008; Orton et al. 2006). These differential responses to ATZ exposure during amphibian development remain to be explained. In amphibians, metamorphosis is stimulated by environmental signals that impinge on the central control of the hypothalamus–pituitary–thyroid axis to initiate release of thyroid hormones [Th; thyroxine (T4), triiodothyronine (T3)] into circulation. Conversion of T4 to T3 and subsequent degradation occur mainly in peripheral tissues and involve deiodinase enzymes (dio). THs then act through thyroid receptors (tr) that regulate gene expression by interacting with the thyroid response element in target genes (Aranda and Pascual 2001). Changes in the expression of *dio* and *tr* could influence the T4 to T3 ratio, which in turn will affect metamorphosis (Manzon and Denver 2004).

We investigated the effects of ATZ on sexual development and metamorphosis in *R. pipiens* under environmentally relevant conditions, as simulated in a mesocosm system. We assessed survival, success and age at metamorphosis, wet weight, snout–vent length (SVL), sex ratio, gonadal histology, gene expression (estrogen- and TH-responsive}
Low atrazine concentrations disrupt Rana pippens development

genes) and enzymatic activities (cyp19 and srd5beta). To our knowledge, this is the first study to use chronic ATZ exposure in amphibians and to evaluate changes in a) cyp19 expression and activity simultaneously; b) etsalpha expression; c) steroidogenic enzyme srd5beta activity; and d) dio and tr expression in premetamorphic and metamorphic tadpoles. Complementary field surveys were undertaken to confirm relevant environmental ATZ concentrations in water and the sex ratio in naturally metamorphosing tadpoles from the same population that we raised in captivity in the mesocosms.

Materials and Methods

Chemicals and reagents. The herbicide AAtrex Liquid 480 (Registration # 18450; purity 97.1% ATZ, 2.9% related triazines, and 5% ethylene glycol wt/vol; Syngenta Crop Protection Canada Inc., Guelph, Ontario, Canada) was purchased locally and used to mimic ATZ input into the environment. We purchased EE2 (CAS 57-63-6, purity ≥98%) from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Animals. Animals were collected in the Raisin River region (Cornwall, Ontario, Canada; latitude, N45°09´58.9´; longitude, W074°47´41.9´). For the field survey (summer 2006), young-of-the-year metamorphs were caught (n = 30) from our reference site [see Supplemental Material, Figure 1 (doi:10.1289/ehp.0901418)]. Animals were brought to the University of Ottawa on ice and anesthetized using a solution of 2% tricaine methanesulfonate (MS-222; Sigma-Aldrich Canada Ltd.). Animals were sacrificed by immersion in 1% MS-222 (Hagen) because tadpoles begin to respond to THs at this stage (Shi 1999). We anesthetized the animals by immersion of the cranial cavity in 1% MS-222 (Hagen) for dual-labeled fluorescent probes), as described in Hogan et al. (2007). Animals were kept in the laboratory until hispanol and stained with hematoxylin and cosin. A blind analysis was performed.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Samples were homogenized at 20 Hz for 2 min. Total RNA from whole brain and liver tissues (from G34 and G42 animals) was isolated using the QIAGEN RNaseasy Micro Kit and RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada), respectively. TRIzol reagent (Invitrogen, Canada Inc., Burlington, Ontario, Canada) was used to isolate total RNA from tadpole tails for both stages. RNA was resuspended in RNase-free water and stored at –80°C. Concentrations of RNA were determined using GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Total CDNA was prepared from 1 µg and 2 µg (from G34 and G42 animal tissues, respectively) of total RNA and 0.2 µg random hexamer primers (Invitrogen) using Superscript II reverse transcriptase (Invitrogen). All procedures followed manufacturer protocols.

We used real-time RT-PCR simplex (SYBR Green detection) and multiplex assays (dual-labeled fluorescent probes), as described by Hogan et al. (2007), to detect transcripts for cyp19, etsalpha, TH receptor isofoms (thalpa and thbeta), deiodinases 2 and 3 (dio2 and dio3, respectively), and the ribosomal protein L8 (rip8). The stress neuropeptide corticotropin-releasing hormone (crh) was also analyzed by RT-PCR in tadpole brains as described by Croteau (2009). Samples were amplified in duplicate along with negative controls (no
template and no reverse-transcriptase controls). Each reaction exhibited an efficiency of 100% ± 10%, with \( r^2 > 0.985. \) Data were normalized to rpL8 mRNA and are presented as fold-change relative to controls.

**Enzyme activity analyses.** We determined enzymatic activity of cyp19 and srd5beta using radiometric methods according to Langlois et al. (2010). The triturated water method was used to assess cyp19 activity in pools of two brains from G42 animals of the same sex, and cyp19 activity is expressed as femtomoles \( ^{3}H \)-H2O per hour \( \times \) milligrams of protein. The activity of srd5beta was determined by the conversion of \( ^{14}C \)-testosterone into \( ^{14}C \)-5beta-reduced metabolites (5beta-DHT and 5beta-androstan-3beta-17beta-diol) in individual tadpole liver at G42 and is expressed as the sum of 5beta-DHT and 5beta-androstan-3beta-17beta-diol per hour \( \times \) milligrams of protein. We measured total protein concentration using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA).

**Statistical analysis.** We used Pearson’s chi-square test to determine statistical differences for sex ratio, survival, and success of metamorphosis. We used one-way analysis of variance (ANOVA) to analyze WW, SVL, AAM, and G34 tadpole gene expression data and two-way ANOVA to analyze G42 tadpole gene expression and enzymatic activity data. ANOVAs were followed by Bonferroni post hoc test (when warranted). Data were tested for normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene’s test). When data failed to meet the assumptions after transformation, we used the non-parametric Kruskal-Wallis one-way ANOVA on ranks, followed by the Mann-Whitney \( U \) test. ATZ and EtOH treatment data were compared with water control, and EE2 treatment data were compared with the EtOH solvent control.

### Results

**ATZ concentrations and mesocosm parameters.** ATZ was detected in every stream sampled on the Raisin River [see Supplemental Material, Figure 1A (doi:10.1289/ehp.0901418)], and concentrations ranged from 0.01 to 1.6 \( \mu \)g/L (see Supplemental Material, Figure 1B). ATZ concentrations in the 0.1- \( \mu \)g/L and 1.8- \( \mu \)g/L ATZ treatment groups were 0.09–0.21 \( \mu \)g/L (nominal 0.1 \( \mu \)g/L ATZ) and 1.6–3.7 \( \mu \)g/L (nominal 1.8 \( \mu \)g/L ATZ), respectively; for details, see Supplemental Material, Figure 2. ATZ concentrations in the control ranged from the LOD (0.003 \( \mu \)g/L) to 0.028 \( \mu \)g/L ATZ (for details, see Supplemental Material, Figure 2). To compensate for water loss through evaporation, groundwater was added regularly to the mesocosm. A small input of ATZ to groundwater coming from peripheral agricultural fields after the spraying season could explain detectable ATZ in the water control on 9 June 2006; however, all control replicates were \(< 0.008 \mu g/L ATZ on 21 July 2006. We found no statistical differences in pH, dissolved oxygen, or temperature measurements among tanks for every monitored event (\( p > 0.05 \); Supplemental Material, Figure 3). Furthermore, at pretreatment, the physicochemical parameters did not vary among treatments and averaged 6.2 mg/L dissolved oxygen and 17.9°C. Results are reported using the nominal concentrations to facilitate presentation.

**ATZ affects metamorphic success.** High survival rates occurred in water (79%) and EtOH (76%) controls (Table 1). The survival rate in the 0.1- \( \mu \)g/L ATZ group was 75%, which was not different from control. However, the 66% survival rate in the 1.8- \( \mu \)g/L ATZ group was significantly lower than control (\( p < 0.05 \)). The EE2 group also exhibited a significant decrease in survival rate (65%) compared with its EtOH control (76%; \( p < 0.05 \)). Significantly fewer ATZ- and EE2-treated tadpoles reached metamorphosis (data were corrected for mortality). In the controls, 76% water control and 85% EtOH control tadpoles reached metamorphosis, whereas 45%, 50%, and 55% completed metamorphosis in 0.1 \( \mu \)g/L ATZ; 1.8 \( \mu \)g/L ATZ, and EE2 treatments, respectively (\( p < 0.001 \)). We found no significant

### Table 1. Effects of ATZ and EE2 on R. pipiens development and metamorphosis.

| Treatment       | n   | Survival (%) | Metamorphic success (%) | AAM (days) | SVL (mm) | WW (µg/L) |
|-----------------|-----|--------------|-------------------------|------------|----------|-----------|
| H2O control     | 150 | 118          | 90                      | 71.5 ± 1.5 | 17.9 ± 0.3 | 0.96 ± 0.06 |
| 0.1 µg/L ATZ    | 150 | 113          | 50*                     | 78.8 ± 2.9 | 17.4 ± 0.1 | 0.89 ± 0.03 |
| 1.8 µg/L ATZ    | 150 | 99*          | 47*                     | 74.9 ± 4.0 | 17.8 ± 0.5 | 0.98 ± 0.06 |
| EtOH            | 150 | 114          | 99                      | 76.1 ± 3.6 | 17.9 ± 0.4 | 1.01 ± 0.04 |
| 1.5 µg/L EE2    | 150 | 97**         | 52**                    | 75.8 ± 4.3 | 16.9 ± 0.4 | 0.83 ± 0.05** |

*Includes all animals that reached or passed G42. **Includes only the animals at G42. \( * p \leq 0.05 \) compared with the water control, and \( ** p \leq 0.05 \) compared with the EtOH control, using either the chi-square test or the one-way ANOVA test.

### Table 2. Gonadal gross morphology and histologic analysis of R. pipiens from the mesocosms and from the Raisin River.

| Treatment       | n   | Sex ratio | TO (%) |
|-----------------|-----|-----------|--------|
| H2O control     | 60  | 37        | 23     |
| 0.1 µg/L ATZ    | 34  | 19        | 15     |
| 1.8 µg/L ATZ    | 31  | 13        | 18     |
| EtOH            | 66  | 44        | 24     |
| 1.5 µg/L EE2    | 35  | 18        | 17     |
| RR reference site | 30 | 20        | 10     |

Abbreviations: F, female; M, male; RR, Raisin River; TO, testicular oocytes. Data represent sample size (n) and sex ratio (male to female ratio) of G42 frogs in all five treatments and in wild-caught metamorphs from reference site R [see Supplemental Material, Figure 1 (doi:10.1289/ehp.0901418)]. From the metamorphosed animals, only G42 individuals were used for gonadal gross morphology. Randomized subsamples of males were chosen for gonadal histology. \( * p \leq 0.05 \) compared with the 0 \( \mu g/L \) ATZ control, and \( ** p \leq 0.05 \) compared with the EtOH control, using the chi-square test.

### Figure 1. Effect of chronic ATZ and EE2 exposures on brain cyp19 expression and enzyme activity in R. pipiens (G42 metamorphs) as determined by real-time RT-PCR. The levels of cyp19 mRNA are expressed relative to the water control group and are normalized to the expression of rpL8 (bars; left y-axis). The activity of cyp19 was assessed using radiometric method and is expressed in femoles/hr normalized to protein content (lines; right y-axis). Values represent mean ± SE (n = 6–8). \( * p \leq 0.05 \) for mRNA differences, and \( ** p \leq 0.05 \) for activity differences, compared with controls by two-way ANOVA followed by Bonferroni post hoc comparisons.
effects of treatment on AAM, SVL, and WW, except that EE2-exposed animals were 5.6% smaller in length and 18% lower in weight, on average, than the EtOH control (p < 0.05). There was no effect of treatment on brain cyp19 mRNA levels (data not shown).

**ATZ induces female-biased sex ratio.** The sex ratios of surviving metamorphs for control groups were 1:0.6 (male:female) and 1:0.6 in the water and EtOH controls, respectively (Table 2). These ratios are comparable with wild-caught metamorphosing animals (1:0.5) from our reference site where we collected eggs for the mesocosm experiment that year. Only the highest ATZ exposure (1.8 µg/L ATZ) significantly altered sex ratio to 1:0.8. Exposure to EE2 did not change the sex ratio (1:0.9); however, histologic analysis indicates that 22% of EE2-treated animals expressed an intersex condition (Table 2). In contrast, we found no intersex gonads in field-collected, water control, EtOH control, or ATZ-treated animals.

**ATZ did not induce cyp19 gene expression and activity.** ATZ did not significantly affect cyp19 mRNA levels in the brains of G42 animals (Figure 1). In contrast, EE2 significantly increased both cyp19 mRNA levels (p < 0.01) and activity (p < 0.001) in the female phenotype animals with treated animals. EE2 exposed to 0.1–100 µg/L ATZ also displayed a dose-dependent increase in cyp19 activity. Conversely, other studies reported no bias in X. laevis sex ratio after chronic ATZ treatments (Carr et al. 2003; Klosa et al. 2009). In addition to female-biased sex ratio, studies have shown that ATZ increases the incidence of intersex condition in amphibians (X. laevis, R. pipiens (Hayes et al. 2002a)). However, we did not observe intersex gonads in ATZ-exposed R. pipiens. These differences between studies may be associated with differences in experimental designs (e.g., different species, stages at exposure, duration of exposure, and other exposure conditions). In contrast, 22% of our EE2-treated male tadpoles displayed testicular oocytes. These results from outdoor mesocosm exposures confirmed a previous study in which chronic exposure of R. pipiens tadpoles to 1.5 µg/L EE2 in a static renewal system resulted in 30% of R. pipiens metamorphs exhibiting an intersex condition (Hogan et al. 2008). Thus, we can say that the experimental mesocosm design functioned successfully as a control system, and our population of R. pipiens has the capacity to respond to estrogenic compounds. Many attempts have been made to investigate possible estrogenic mechanisms of ATZ action in several vertebrate models. ATZ failed to induce estrogen-mediated responses in the uterus of immature female Sprague-Dawley rat in the estrogen-responsive MCF-7 human breast cancer cell line, and in the estrogen-dependent recombinant yeast strain PL3 (Connor et al. 1996). Moreover, ATZ also failed to induce vitellogenin production in vivo in X. laevis liver and in vitro in X. laevis hepatocyte cultures after exposure to levels ranging from 0.1 to 100 µg/L ATZ (Oka et al. 2008). Because ovarian differentiation in amphibians is mediated by estrogens, the dominant hypothesis in the literature remains that ATZ induces cyp19 activity (Hayes et al. 2002a, 2003; Taver-Mendoza et al. 2002a, 2002b). There is evidence that cyp19 activity is induced indirectly through phosphodiesterase inhibition (Roberge et al. 2004) and through binding to steroidogenic factor 1 (Fan et al. 2007). Whether induction of cyp19 activity is the only estrogenic action of ATZ is still a matter of debate. Several studies have refuted the cyp19 induction hypothesis in amphibians (Crady et al. 2005; Hecker et al. 2005a, 2005b; Oka et al. 2008). Our data also support that ATZ action is not mediated via cyp19 activity induction, because we detected no changes in cyp19 mRNA levels or a cyp19 enzyme activity in R. pipiens tadpole brain. Taken together, the cyp19 hypothesis for ATZ disruption of sexual development is refuted.

**Figure 2.** Effect of chronic ATZ and EE2 exposures on the expression of brain eralpaha (A) and tail dio3 (B) in R. pipiens determined by real-time RT-PCR on metamorphic G34 tadpoles that failed to metamorphose. The mRNA levels are expressed relative to the water control group (0 µg/L ATZ) and are normalized to the expression of gapdh. *p < 0.05 compared with controls by one-way ANOVA followed by Bonferroni post hoc comparisons.

**Figure 3.** Effects of chronic ATZ exposure on liver srdbeta activity in R. pipiens determined at G42 using the radiometric method and expressed in pmol/hr (srdbeta) normalized to protein content. Values represent the mean ± SE (n = 6). *p < 0.05 by two-way ANOVA followed by Bonferroni post hoc comparisons.
not well supported; therefore, we investigated other potential mechanisms of action.

We studied *Xenopus* expression and srd-5beta activity, two pathways that, if altered by ATZ, could produce an estrogen-like response. Our data confirmed that tadpoles exposed to 1.8 µg/L ATZ at G34 expressed higher *Xenopus* mRNA levels in brain compared with control animals. Similar increases in *erpalhida* expression in R. *pipiens* have been reported after EE2 treatment under laboratory conditions (Duarte et al. 2006). In our mesocosm study, EE2 doubled the brain *erpalhida* mRNA level. This suggests that estrogenic compounds have the ability to increase *erpalhida* mRNA levels. Hogan et al. (2007) reported that expression of brain *erpalhida* mRNA was similar at G30 and G36, a period that coincides with gonadal differentiation. It is therefore possible that a 2.5-fold increase in *erpalhida* expression at G34 after ATZ exposure could alter the sensitivity of the developing brain to estrogen and lead to other physiologic changes. R. *pipiens* tadpoles are most sensitive to EE2-induced feminization early in development, before G30 (Hogan et al. 2008); therefore, amphibians exposed to ATZ in early development may be more sensitive to ATZ-induced feminization.

We also demonstrated that ATZ alters hepatic srd-5beta activity in R. *pipiens* tadpoles. Recent studies in our laboratory have shown that inhibiting srd-5beta activity resulted in a female-skewed sex ratio, suggesting that this enzyme could be involved in amphibian gonadal development (Duarte-Guterman et al. 2010). In mammals, a natural sexual dimorphism exists in hepatic srd-5beta activity; for example, female rat liver contains more srd-5beta activity than male liver (Cook GM, unpublished data). In the present study, we observed a similar dimorphic pattern in the livers of H2O-control tadpoles. However, after exposure to ATZ, this sex difference in srd-5beta activity disappeared. There is a lack of data regarding the importance of this sexual dimorphism, but it likely results in differential androgen status in developing males versus females. In addition to a possible role in gonadal development, srd-5beta is also involved in other biological functions such as erythropoiesis (Garavini and Cristofori 1984) and bile biosynthesis (Kondo et al. 1994); thus, srd-5beta alteration could also lead to other physiologic defects. Future studies should explore these new end points for ATZ action.

In amphibians, THs are essential for metamorphosis and are involved in the remodeling of TH target tissues such as brain, hindlimb, intestine, and tail (Shi 1999). A disruption in TH production can result in important physiologic defects. Developmental exposure of *X. laevis* to ammonium perchlorate (an inhibitor of thyroid iodide uptake) resulted in fewer tadpoles completing tail resorption, forelimb emergence, and hindlimb development (Goleman et al. 2002). Several studies have suggested that ATZ alters the thyroid axis in *X. laevis* (Freeman and Rayburn 2005) and rats [female albino rats (Kornilovskaya et al. 1996); male Wistar rats; (Stoker et al. 2000)]. Here, we present evidence that ATZ exposure alters the thyroid axis by affecting success of metamorphosis and also TH-related gene expression in *R. pipiens*. Our real-time RT-PCR results indicate a 79% reduction of *dio3* mRNA in G34 tadpole tails. This decrease in mRNA level is most likely caused by a compensatory mechanism of the animals to trigger metamorphosis by reducing T3 breakdown to inactive metabolites.

**Conclusions**

Using an outdoor mesocosm design, we found that ATZ can affect amphibian development at levels measured in water across the distribution of *R. pipiens* in North America (Graymore et al. 2001). Much controversy surrounds the effects of ATZ on frogs. For example, at one extreme, *X. laevis* exposed to low levels of ATZ, under laboratory conditions suffered gonadal dysgenesis (Hayes et al. 2006), whereas at the other extreme, no effects were observed after similar exposures (Coady et al. 2005; Klos et al. 2009). The reasons for such differences are numerous and have been discussed previously (Hayes 2004; Solomon et al. 2008), and there is the question of the relevance of studies with nonnative species to predict potential effects on indigenous species. We used a mesocosm study to directly test the effects of a commercial ATZ preparation on a North American native species. Our results using mesocosms are somewhat intermediate compared with previous laboratory and field studies. Nevertheless, the present study demonstrates that ATZ can be biologically active in *R. pipiens*, as we report female-biased sex ratios and disruption of metamorphosis with associated changes in gene expression after ATZ exposure. These responses occurred with environmentally relevant exposure conditions. Female-biased sex ratio and disruption of metamorphosis are important physiologic consequences of this exposure, which could potentially alter amphibian population fitness. Therefore, subsequent studies should examine population-level effects associated with the widespread use of ATZ with particular focus on risks to native amphibian populations.

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