There Is No Denying This: Defusing the Confusion about Atrazine

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Recent studies from my laboratory, showing the chemical castration (demasculinization) and feminization of amphibians by low but ecologically relevant concentrations of atrazine in the laboratory and in the wild, prompted a critical response from atrazine's manufacturer, Syngenta Crop Protection, and Syngenta-funded scientists. A careful analysis of the published data funded by Syngenta, and of several studies submitted to the US Environmental Protection Agency (EPA) by the Syngenta-funded panel for data evaluation, indicates that the data presented in these studies are not in disagreement with my laboratory's peer-reviewed, published data. Further, the published and unpublished data presented to the EPA by the Syngenta-funded panel (and touted in the popular press) suffer from contaminated laboratory controls; high mortality; inappropriate measurements of hormone levels in stressed, sexually immature animals during nonreproductive seasons; and contaminated reference sites. The confounding factors in the industry-funded studies severely limit any conclusions about the adverse effects of atrazine on amphibians and prevent meaningful comparisons with my laboratory's published data.

Keywords: atrazine, amphibian, endocrine disruptor, chemical castration, feminization

Years have passed since DDT was banned in the United States, but it is unclear how much policymakers and the public have learned from the case of this dangerous pesticide. DDT was banned on the basis of even less scientific evidence than currently exists for the negative impacts of atrazine. Atrazine, an herbicide, is the top-selling product for the largest chemical company in the world. Its primary consumer (the United States) boasts the largest economy in the world, and it is used on corn, the largest crop in the United States. One of the primary targets for atrazine is the weed common groundsel (*Senecio vulgaris*), the most widespread botanical in the world (Kadereit 1984). As a result of its frequent use, atrazine is the most common contaminant of ground, surface, and drinking water (Aspelin 1994), and its use over the last 40 years has resulted in the evolution of more herbicide-resistant weeds (> 60 species) than any other herbicide (Heap 1997, Gadamski et al. 2003). Given its status, it is no surprise that the results of recent studies from my laboratory—showing that atrazine is an endocrine disruptor that demasculinizes and feminizes male amphibians at low but ecologically relevant doses—sparked debate (Carr and Solomon 2003). Our work potentially linked two of the most debated issues in conservation and environmental biology: the causes of amphibian declines (Green 2003, Schmidt 2003, Storfer 2003) and the potential impact of endocrine disruptors in the environment (Colborn 1994, Ankley et al. 1997, Fenner-Crisp 1997, MRC IEH 1999, Harvey and Johnson 2002). The controversy emanated from members of the Ecorisk Atrazine Endocrine Ecological Risk Assessment Panel, a group funded by the manufacturer of atrazine, Syngenta Crop Protection, through the consulting company Ecorisk, Inc. Because I was a former member of this panel, my laboratory's work was the main target of its criticism, but in fact we were not the first to show the effects of atrazine on gonadal development in the African clawed frog (*Xenopus laevis*) in the laboratory (Tavera-Mendoza et al. 2002), nor were we the first to show the effects of atrazine on gonadal development in wild amphibians (Reeder et al. 1998).

Of the many emerging problems in science, endocrine disruption has generated some of the largest debates (Colborn 1994, Fenner-Crisp 1997). Endocrine disruption, defined as interference with hormone synthesis, secretion, receptor binding, activity, or degradation, was formerly recognized as a concern in ecotoxicology by the US Environmental Protection Agency (EPA) with the creation of the Endocrine Disruption Screening and Testing Advisory Committee in 1996, and a number of compounds have been shown to produce such effects. Pharmaceuticals such as ethynylestradiol, used in birth control pills, can have feminizing effects on wildlife (Desaulniers et al. 2003, Kirigaya et al. 2003, Naciff

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et al. 2003); chemicals used in the production of plastics, such as bisphenol A, can be estrogenic (Gebhard et al. 1997, Diel et al. 2004, Masutomi et al. 2004, Takagi et al. 2004, Terasaki et al. 2004); and even natural compounds, such as the plant phytoestrogens, are of concern in some cases (Cabanes et al. 2004, Christensen and Lephart 2004, Takagi et al. 2004, Vaya and Tamir 2004). In addition, a large number of pesticides may have endocrine-disrupting effects. Two well-known cases are the antifouling agent tributyltin (TBT), which is responsible for the induction of intersex development in mollusks (specifically imposex, or the development of male and female gonads in a single individual; Marshall and Rajkumar 2003, Reitsema et al. 2003, Stickle 2003, Horiguchi et al. 2004), and DDT, which (along with its metabolites) has a number of endocrine-disrupting effects, including estrogen receptor agonism (Noriega and Hayes 2000, Leanos-Castaneda et al. 2002, Tollefsen et al. 2002), inhibition of prostaglandin synthesis (Lundholm and Bartonek 1991), and androgen receptor antagonism (Daxenberger 2002). DDT and TBT, however, were regulated long before there was an EPA Endocrine Disruption Screening and Testing Advisory Committee, before the term "endocrine disruption" was even in use, and before the mechanisms or even the full effects of these pesticides on nontarget organisms were recognized (in fact, the regulation of TBT occurred after only three peer-reviewed papers on its effects, and all were field-based observations).

Unlike the case with DDT and TBT, scientists already know a great deal about the endocrine-disrupting effects of atrazine. When I was invited to join the (then Novartis-funded) Ecorisk Atrazine Endocrine Ecological Risk Assessment Panel in 1997, and when I accepted funding to examine the effects of atrazine on amphibians in 1998, it was already established that atrazine exposure resulted in elevated estrogen (Eldridge et al. 1994a, 1994b, Stevens et al. 1994, Wetzel et al. 1994) and was associated with mammary tumors in rodents (Eldridge et al. 1994b, Stevens et al. 1994, Tennant et al. 1994, Wetzel et al. 1994). It was also well known that atrazine was associated with decreased androgens, with inhibition of androgen action (Kniewald et al. 1979, 1980, 1995, 2000, Babic-Gojmerac et al. 1989, Simic et al. 1991, 1994, Friedmann 2002a), and with decreased fertility (i.e., chemical castration in rodents; Simic et al. 1994, Kniewald et al. 1995, Friedmann 2002a). Furthermore, one panel member (John Giesy, professor at Michigan State University and former graduate advisor of the current vice president of Syngenta, Gary Dickson) coauthored two papers showing that atrazine induced the production of aromatase (the enzyme that converts androgens to estrogens) in a human cell line (figure 1; Sanderson et al. 2000, 2001). It was this work, in fact, that warned, "Exposure to triazine herbicides, which are produced and used in large quantities, and are ubiquitous environmental contaminants, may similarly contribute to estrogen-mediated toxicities and inappropriate sexual differentiation" (Sanderson et al. 2000, p. 126), and it was this concern that prompted Syngenta and Ecorisk to contract with my laboratory to examine the endocrine-disrupting effects of atrazine in amphibians.

Laboratory studies were initially conducted using *X. laevis* to examine the effects of atrazine on metamorphosis, laryngeal growth, and gonadal differentiation, end points chosen to address the disruption of thyroid hormone, estrogen, and androgen function, respectively. Under the auspices of the Ecorisk panel, and sponsored by Syngenta, my laboratory showed that male larvae exposed to at least 1 part per billion (ppb) atrazine throughout development suffered from impaired laryngeal growth. This demasculinization of the larynx suggested that atrazine reduced androgens in (i.e., chemically castrated) exposed males, because laryngeal growth is androgen dependent (Tobias et al. 1991, 1993, Robertson et al. 1994). Although we took note of ambiguous gonads at the time, the full effect of atrazine on the gonads was not realized immediately. These data were provided to our sponsor as early as 1999, but were not reported to the EPA until 2000 (Parshley 2000), and to date remain unpublished in the open literature.

Once my laboratory repeated this work independent of the Ecorisk panel, it was subsequently published (Hayes et al. 2002a). We repeated our initial findings on the larynx in animals from two sources, and it was during these studies that we discovered the effects of atrazine on the gonads: Atrazine at levels of 0.1 ppb or higher produced gonadal deformities, including multiple testes, nonpigmented ovaries (or what appeared to be ovaries), and hermaphrodites (Hayes et al. 2002b). Although we did not present the frequencies of each type of gonadal deformity (the data were reported as the frequency of total deformities), none of these morphologies occured in unexposed animals (> 10,000 observations). Apparently these effects occurred only in males (i.e., the hermaphrodites were genetic males with ovaries and not genetic females with testes). We also showed that males were in fact chemically castrated when exposed to atrazine (Hayes et al. 2002a), and we have subsequently shown that this effect occurs at doses as low as 0.01 ppb in adult males. We and an independent laboratory in Japan showed that atrazine indeed induced expression of the aromatase gene (CYP19), and this gene is associated with gonadal malformations 100 percent of the time (Miyahara et al. 2003). These data all support the

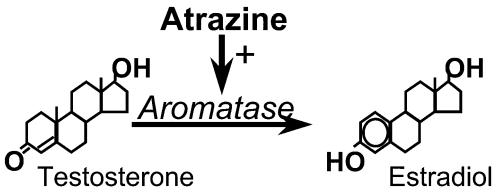

Figure 1. Proposed mechanism of action of atrazine effects in amphibians. Atrazine induction of aromatase has been verified in all vertebrate classes examined. Animals are chemically castrated (demasculinized) as a result of the loss of testosterone (aromatase substrate) and feminized as a result of the inappropriate synthesis of estrogens in males.

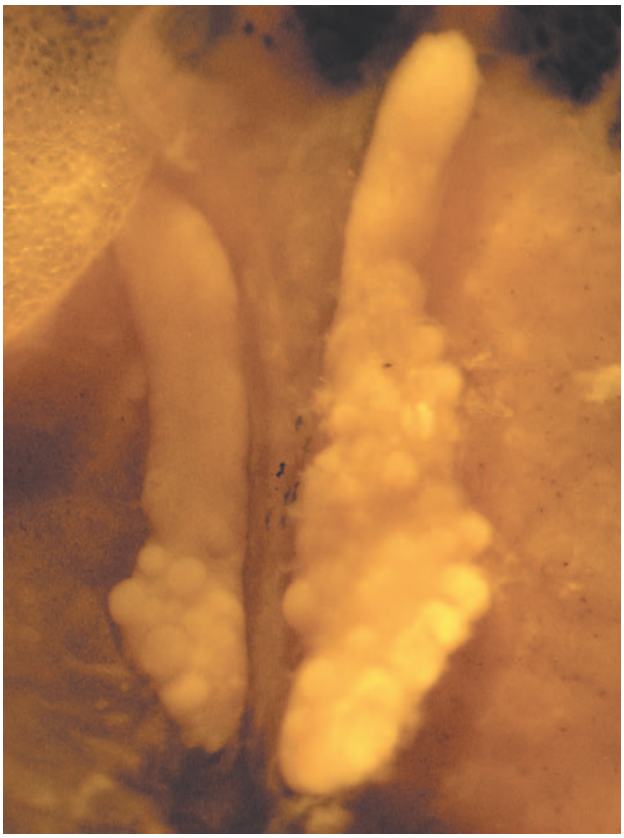

Figure 2. Gonads from a male leopard frog (*Rana pipiens*) exposed to 0.1 parts per billion of atrazine in the laboratory. The animal is clearly male (testes are evident), but vitellogenic oocytes are observed protruding through the posterior surface of the testes. Yellow color is the result of fixation in Bouin's solution. Source: Hayes et al. 2000b.

hypothesis that atrazine's assault on male sexual development is due to the induction of aromatase, consistent with findings in mammals gathered primarily by industry and industry-funded scientists (Eldridge et al. 1994a, 1994b, Stevens et al. 1994, Tennant et al. 1994, Wetzel et al. 1994, Trentacoste et al. 2001), although I acknowledge that atrazine acts as an endocrine disruptor through many other well-documented mechanisms (Babic-Gojmerac et al. 1989, Simic et al. 1991, Cooper et al. 1999, 2000, Cummings et al. 2000, Kniewald et al. 2000, Narotsky et al. 2001, Trentacoste et al. 2001, Bisson and Hontela 2002, Friedmann 2002b).

Studies in male leopard frogs (*Rana pipiens*), as in *X. laevis,* showed that ecologically relevant doses of atrazine (≥ 0.1 ppb) produced gonadal abnormalities. Atrazine exposure resulted in testicular oocytes in *R. pipiens.* This effect occurred at a maximum frequency of 29 percent in males exposed to 0.1 ppb, and in some cases oocytes were vitellogenic (figure 2), consistent with findings that atrazine-exposed males produce vitellogenin (McCoy et al. 2002, Miyahara et al. 2003). Using this end point, we examined *R. pipiens* across the United States in a transect that extended from Utah to the Iowa–Illinois border. Animals were selected from a variety of habitats, including golf courses, wildlife management areas, rivers, and agricultural runoff from cornfields, among others. Atrazine levels were measured by three independent laboratories, and sites where atrazine levels were less than 0.1 ppb (the threshold concentration) were designated reference sites, whereas all sites with levels of 0.1 ppb or more were considered exposed. Water samples for analysis were taken at the same time that newly metamorphosed frogs (100 from each site) were collected. At the time the water samples were taken (late July–early August), atrazine levels were low, so measurements of contamination were actually underestimates. Males with testicular oocytes, identical to the morphology produced by atrazine exposure in the laboratory, were found at every site where atrazine contamination was detected. Although other contaminants (or stressors) may have been present at the sites, we concluded that the close association between atrazine contamination and hermaphroditism in the wild, in combination with support from controlled laboratory studies, suggested a cause–effect relationship between atrazine exposure and gonadal abnormalities. This conclusion was supported by the previously reported association between testicular oocytes and atrazine contamination (P = 0.07) in the northern cricket frog (*Acris crepitans*). Furthermore, because similar effects or associations were observed in three frog species from different genera (and different families: Pipidae, Ranidae, and Hylidae), we concluded that this effect of atrazine could be generalized to anurans and not restricted to a single (or a few related) species.

Ecorisk conducted several studies on amphibians after I left the panel in November 2000. Although the results of these studies had not yet been published in the peer-reviewed literature, Alan Hosmer, manager of ecological sciences at Syngenta, reported in 2003 that Syngenta had "recently provided the EPA with numerous new scientific studies that will assist in understanding the uncertainties surrounding the possible risks of atrazine to amphibians and other wildlife species" (Hosmer 2003). Several studies conducted by the panel (Hecker et al. 2003a, 2003b, Sepulveda and Gross 2003, Smith et al. 2003a, 2003b) became available in the public record following submission to the EPA (Steeger et al. 2003a, 2003b, 2003c, 2003d, 2003e) and presumably represent the sum of studies referenced in press releases and by Carr and Solomon (2003). I will review the Ecorisk studies here, along with the only peer-reviewed publication (Carr et al. 2003) at the time of Hosmer's announcement.

At least two laboratory studies on *X. laevis* were conducted by the Ecorisk panel, one at Texas Tech University (under the direction of James Carr) and one at Michigan State University (under Giesy's direction). Like the studies performed by my laboratory, the study by Carr and colleagues (2003) showed that atrazine exposure resulted in gonadal abnormalities. Hermaphrodites and males with multiple testes were produced in their study, with morphologies identical to morphologies published in our earlier study (figure 3; Hayes et al. 2002a, Carr et al. 2003). Although the authors did not

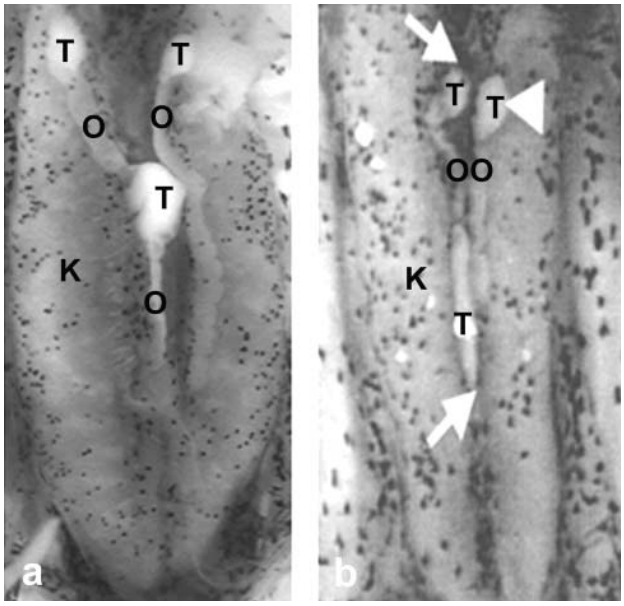

Figure 3. (a) Mixed hermaphrodite produced by atrazine exposure (0.1 parts per billion) in a study by Hayes and colleagues (2002a) compared with (b) similar gonadal abnormalities produced in atrazine-exposed animals in a study by Carr and colleagues (2003). Both photographs show animals with three testes and multiple ovaries. Abbreviations: K, kidney; O, ovary; T, testis.

report the incidence of nonpigmented ovaries, they reported hermaphrodites and animals with single-sex polygonadism (described as "discontinuous gonads") at frequencies similar to or higher than the incidences that we observed. This observation is true regardless of whether true doses (micrograms [µg] atrazine per tadpole) or atrazine concentrations (µg per liter [L]) are compared (figure 4). Further, Carr and colleagues (2003) showed that the effect of atrazine on the gonads was very robust, with *P* values of 0.0003 and 0.0042 for single-sex polygonadism (discontinuous gonads) and hermaphroditism, respectively. In addition, in the initial report the authors described a host of other highly significant adverse effects of atrazine, including abnormal swimming ($P = 0.004$), edema ($P = 0.02$), inhibition of foreleg emergence ($P = 0.03$), inhibition of tail reabsorption ($P = 0.04$), and changes in laryngeal size ($P = 0.03$). In the second laboratory study on *X. laevis,* at Michigan State University (Hecker et al. 2003b), no significant effects of atrazine were reported, and it was suggested that hermaphroditism could occur in *X. laevis* in the absence of atrazine (e.g., in controls). The same laboratory examined the effects of atrazine exposure on testosterone levels. They reported that their findings did not support our previous report that atrazine chemically castrated exposed *X. laevis,* and aromatase expression was not detected.

In a field-based study in South Africa (DuPreez 2003), the Ecorisk panel suggested that hermaphroditism was not due to atrazine and was a natural phenomenon. In this study, they

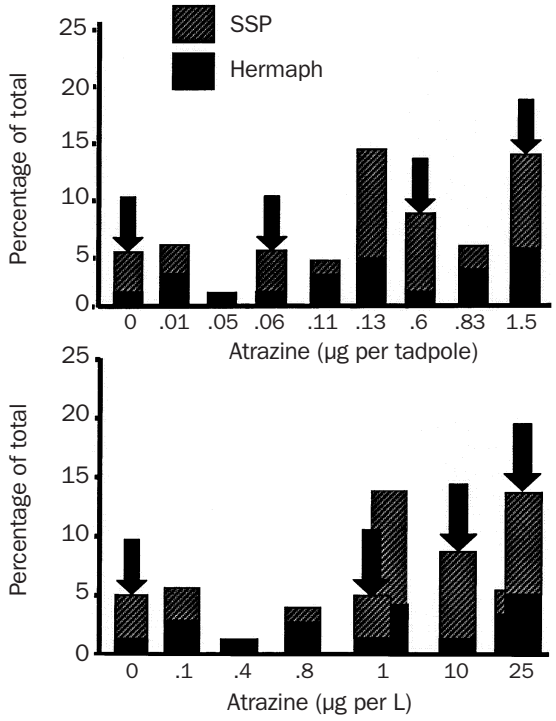

Figure 4. Frequency of animals with single-sex polygonadism (SSP; previously described as multiple, lobed, or broken testes) and hermaphroditism produced by atrazine exposure in the study by Hayes and colleagues (2002a) compared with the frequency observed by Carr and colleagues (2003). Data are shown for both the dose (micrograms [µg] atrazine per tadpole, top panel) and the concentration (µg atrazine per liter, bottom panel). Arrows indicate data from Carr and colleagues (2003).

examined *X. laevis* of various ages collected from six ponds. Three of the ponds were in corn-growing regions, and three were in non-corn-growing regions. Because hermaphroditic *X. laevis* were collected at all six ponds, the authors concluded that atrazine could not be the cause. The Ecorisk panel also examined the effects of atrazine in green frogs (*Rana clamitans*) in the laboratory, for comparison with my laboratory's work on *R. pipiens.* Their study concluded that atrazine showed no effects on mortality, metamorphosis, or sex differentiation, again reportedly refuting our findings in *R. pipiens.*

It was presumably the results of these studies that prompted the industry-funded panel's response to our published work. Although the results were not yet peer-reviewed or even submitted to the EPA at the time, the Syngenta press release quoted James Carr as saying, "We have been unable to reproduce the low-concentration effects of atrazine on amphibians reported elsewhere in the scientific literature" (Kendall et al. 2002). The statement attributed to Carr is particularly puzzling, because once the data were published, Carr's results actually supported our findings (Carr et al. 2003). Despite the significance of their findings on the gonads,

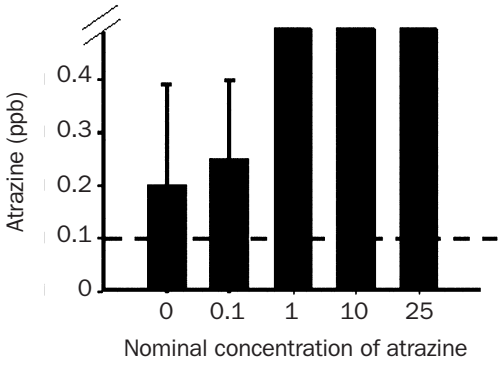

Figure 5. Atrazine levels in control and atrazine-treated replicates in the* Xenopus laevis *study funded by Syngenta Crop Protection and conducted under the supervision of John Giesy at Michigan State University (Smith et al. 2003a, Hecker et al. 2004). Dashed line shows 0.1 part per billion, the threshold dose for the induction of hermaphroditism by atrazine. Note that the control groups are contaminated with atrazine in excess of twofold above threshold. By contrast, in the studies performed by my laboratory, atrazine was never detected in controls, and actual atrazine concentrations in treatment groups were always within 10 percent of the nominal doses (Hayes et al. 2002a, 2002b, 2002c).

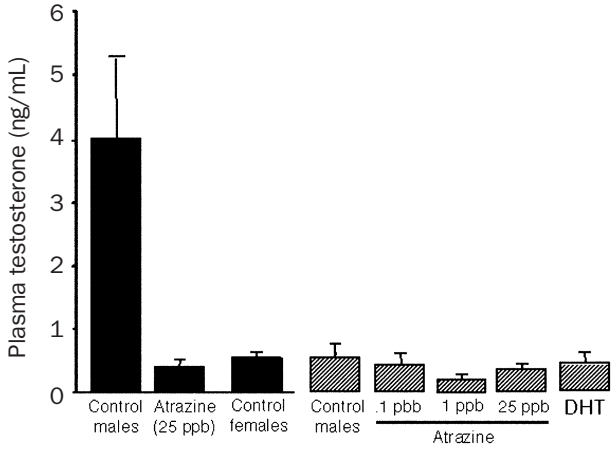

Figure 6. Plasma testosterone levels for male and female control animals and for atrazine-exposed adult males, as reported by Hayes and colleagues (2002a) (shown in black), compared with the levels reported by Hecker and colleagues (2003b) (hatched bars). Note that none of the hormone levels in control, atrazine-treated, or even androgen-treated animals measured by Hecker and colleagues (2003b) differed from those reported by Hayes and colleagues (2002a) in atrazine-treated males or normal females. Abbreviation: DHT, dihydrotestosterone.

with reported probabilities exceeding 99 percent that the effects were due to atrazine exposure, and with several other adverse effects supported with *P* values less than 0.05, the authors referred to many of these highly significant effects as "weak trends" and reported that our work was not repeatable.

There were many problems associated with the husbandry in Carr and colleagues's study (2003). As many as 60 percent of the animals did not metamorphose in some treatments, and surviving animals suffered from retarded growth (animals that metamorphosed later were smaller than animals that metamorphosed earlier, the reverse of what is expected). Further, the authors incorrectly compared the doses (μg atrazine per tadpole) in their study with the doses used in my laboratory's study. The concentrations were similar, but during critical developmental stages, the doses were 1/8 the doses used in our study, because the tadpoles were overcrowded—60–65 tadpoles per 2 L (30.0–32.5 tadpoles per L) compared with 30 tadpoles per 4 L (7.5 tadpoles per L) in our studies—and because only half of the atrazine was renewed during water changes.

The other studies conducted by the Syngenta-funded panel were reviewed but remained unpublished. Their data and interpretations are available through the EPA, however (Hecker et al. 2003a, Sepulveda and Gross 2003, Smith et al. 2003a, 2003b, Steeger et al. 2003a, 2003b, 2003c, 2003d). In its evaluation of the Michigan State University study, the EPA noted that "the negative controls were contaminated with atrazine at levels comparable to those in the 0.1 μg/L atrazine treatment" (Steeger et al. 2003e). In fact, at times the atrazine levels in the control groups exceeded the threshold dose more than fourfold (figure 5; Hecker et al. 2003b, Giesy et al. 2004). Thus, the claims that hermaphrodites developed in control groups, independent of atrazine, are unsubstantiated. Because the authors changed only half of the rearing medium every three days, each detection of atrazine at 0.4 ppb would mean that animals were exposed to atrazine at levels of 0.1 ppb or higher for nine days. In our studies, atrazine (measured independently by PTRL West, the same company contracted by Syngenta) was never detected in controls, and actual atrazine concentrations were always within 10 percent of the nominal doses (Hayes et al. 2002a, 2002b, 2002c).

It is not clear why the controls were contaminated with atrazine in the Michigan State University study. The water source at Michigan State may be contaminated, the researchers may not have taken care to avoid cross-contamination during the experiment, or the aeration and volatilization of atrazine may have resulted in contamination of control tanks that were uncovered and maintained adjacent to similarly uncovered experimental tanks. In addition, because the tanks were uncovered, "animals may have hopped between treatments," according to EPA evaluations (Steeger et al. 2003e), and in some cases leaped completely out of the experiment. Thus, there were many flaws associated with this study. As the EPA evaluation pointed out, "a combination of tank effects, contaminated controls, high variability and an apparent lack of responsiveness to estradiol made it difficult for the study authors to test their hypothesis and to differentiate treat-

ment effects" (Steeger et al. 2003e), let alone draw the conclusion that atrazine had no effect in the species.

Similarly, this same laboratory's report that they were unable to reproduce my laboratory's findings that testosterone levels were decreased in atrazine-exposed male *X. laevis* (Hecker et al. 2003b) is questionable. Testosterone levels in atrazine-exposed animals in the Michigan State study were comparable to the levels in animals castrated by atrazine in our initial study (figure 6; Hecker et al. 2003b). The conclusion that atrazine had no effect, however, was based on similar testosterone levels in the atrazine-treated males and the controls. Thus, the study did not show that atrazine-treated animals had normal testosterone levels; rather, none of the treatment groups (including controls) had normal levels (figure 6). Even adding androgen (dihydrotestosterone, DHT) to the treatment water did not raise androgen levels above normal. A careful analysis of this study reveals that androgen levels were measured in juvenile animals from blood samples taken in the daytime (*X. laevis,* like most frog species, are nocturnal). Furthermore, animals were sampled in the winter (they are active in spring and summer), were stressed by subjection to anesthesia for at least two hours before sampling, and were maintained in the laboratory with the atrazine-contaminated water. Thus, again, the poor design, contamination, and lack of care in this study render the claims of "no effect" highly questionable.

The study conducted at Michigan State that claimed atrazine had no effect on sex differentiation or mortality in *R. clamitans* (Hecker et al. 2003a) is also highly suspect. As in other studies from this laboratory, the control water contained atrazine in excess of the 0.1-ppb threshold for effects. More important problems occurred in this study, however, which limit its usefulness: Up to 86 percent of the animals died in some treatments (80 percent even in controls) because of inadequate husbandry practices (figure 7; Hayes et al. 2002a, 2002b, 2002c). In my laboratory, mortality is usually less than 10 percent, and anything greater than 15 percent would result in cancellation of the study (Hayes et al. 2002a, 2002b, 2002c). Given that as few as 14 percent of the test animals in the Michigan State University study survived, it is not possible that the authors could evaluate the effects of atrazine on the gonads or sex ratio. Even more disconcerting, the authors claimed that atrazine had no effect on mortality. It is difficult to understand how the authors could ascertain the impact of atrazine on mortality when, on average, 76.5 percent of the test animals (including controls) died as a result of poor husbandry (EPA standards require that 70 to 90 percent survive). The most basic measure of toxicity is the median lethal dose (LD_{50}), the dose at which 50 percent of the test animals die as a result of exposure to the compound. It is not possible to estimate LD_{50} when 80 percent of the unexposed controls have suffered mortality. Therefore, it is inappropriate to estimate developmental effects, such as the induction of hermaphroditism, especially when the frequency of the survival (14 percent in some cases) was only about half the frequency at which gonadal abnormalities are expected to occur (29 percent). Thus, the sample size in this study and the apparent poor condition of the few surviving animals, along with the atrazine-contaminated controls, greatly limit any conclusions regarding sex ratios or effects on gonadal development (or on mortality, for that matter).

Similar problems are apparent in the field study conducted on *X. laevis* by the panel (Du Preez 2003). As mentioned above, the Syngenta-funded panel reported hermaphrodites from both corn-growing and non-corn-growing regions in South Africa, and concluded that atrazine could not be involved in inducing hermaphroditism. First, this study suffered from the problem that the authors examined animals of multiple ages and could not possibly know that the animals examined developed in the ponds where triazines were measured. The authors are incorrect in their assumption that, as aquatic frogs, *X. laevis* do not move between ponds. *Xenopus* not only regularly move across land (during rains) but often breed in temporary bodies of water, such as flooded corn fields (personal observation). Even in the Syngenta-funded study (Du Preez 2003), one of the study ponds was decimated when catfish moved into it and preyed on the frogs. Thus, the authors' claims rely on the unlikely assumption that fish (which are truly aquatic and have no lungs or legs) are capable of moving between ponds, but frogs (which have lungs and legs) are not.

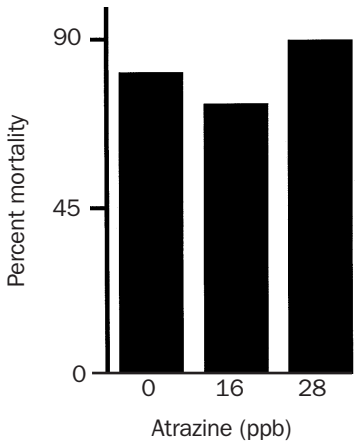

Figure 7. Mortality in control and atrazine-treated green frogs (*Rana clamitans*) in a study performed at Michigan State University and funded by Syngenta Crop Protection (Hecker et al. 2003a, Coady et al. 2004). Nominal doses of atrazine were 10 and 25 parts per billion (ppb). Actual atrazine concentrations were reported as up to 16 and 28 ppb for the 10- and 25-ppb dose, respectively (depending on which reported measurements the authors used), and up to 0.3 ppb in the controls. Note that mortality averaged 76.5 percent across all treatments. In my laboratory, mortality is usually less than 10 percent, and anything greater than 15 percent would result in cancellation of the study (Hayes et al. 2002a, 2002b, 2002c).

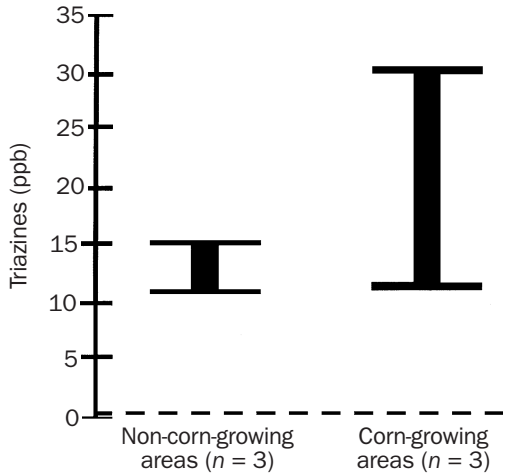

Figure 8. Range of triazine levels in non-corn-growing and corn-growing regions in South Africa, as measured in a study funded by Syngenta Crop Protection (Hecker 2003, Smith et al. 2003a, Hecker et al. 2004). Dashed line indicates the atrazine threshold dose (0.1 parts per billion [ppb]). Note that total triazines exceeded the threshold dose 100-fold, even in non-corn-growing regions. Atrazine levels alone were at least four times above the threshold. (Note that these levels were probably underestimates, as emphasized by the authors.) As a result, the study lacked a meaningful control. By contrast, in the study performed by my laboratory, a single site had atrazine levels below 0.1 ppb (nondetectable), and this site was the only site that lacked animals with gonadal abnormalities (Hayes et al. 2002b, 2002c).

More important, the authors report that triazines were measured at both sites at levels above the threshold for producing hermaphrodites, and, as pointed out in the EPA evaluation, "reference sites all contained measurable residues of atrazine (and other triazines) that were, in some cases, higher than sites considered representative of atrazine exposure" (Steeger et al. 2003b). In fact, total triazines exceeded the threshold at least 100-fold (up to 150-fold), and even atrazine alone exceeded the threshold fourfold in the reference sites (Hecker 2003, Smith et al. 2003a, Hecker et al. 2004; figure 8). Furthermore, the authors pointed out that "frogs living in these dams [ponds] were undoubtedly exposed to much higher atrazine and other triazine levels than had been recorded during the present study" (Smith et al. 2003b, Steeger et al. 2003c). Thus, the study's conclusions that atrazine (and triazines in general) can be ruled out as causes of hermaphroditism are misleading. As in the laboratory studies from the same group, there were no true controls. As the EPA's evaluation states, "Given that atrazine and/or its degradates were present in reference ponds at levels at times equivalent to experimental pond sites and the authors concede that atrazine exposure prior to winter floods was likely higher at all sites, it is unclear how the study can differentiate atrazine effects on frogs at reference and experimental sites" (Steeger et al. 2003b, p. 6). No conclusions regarding the effects of atrazine can be drawn from the South Africa study, because its reference sites contained triazine levels that overlapped (and exceeded) the levels in the experimental groups.

In summary, seven studies have been published to date that show effects of atrazine on amphibian sexual development. Although conducted under different experimental conditions, these studies support the conclusion that atrazine is a potent endocrine disruptor that both chemically castrates and feminizes male amphibians. The confusion generated by Syngenta's press releases and statements to the popular press has not been substantiated by peer-reviewed science. Furthermore, as described here, the studies made available to the EPA (Steeger et al. 2003a, 2003b, 2003c, 2003d, 2003e) and recent publications (Coady et al. 2004, Hecker et al. 2004) have not supported Syngenta's claims. Unfortunately, financial incentives and industry involvement in the research on this issue have generated confusion in the scientific community and the public sector, making it more difficult to understand the science involved.

The influence of industry can be demonstrated most clearly, and perhaps most objectively, by examining the path associated with negative findings. To this end, I examined 16 experiments that tested the effects of atrazine on gonadal development in anurans (table 1). The studies represented reports from peer-reviewed publications, reports to the EPA, and presentations at professional conferences or symposia (published abstracts); they included 10 laboratory studies and 6 field studies, with 7 studies supported by Syngenta and 9 funded by independent sources. They included studies from six independent laboratories from three countries and studies on five species (four anuran families). I grouped these reports into negative studies (reporting no effects) and positive studies (reporting effects on the gonads of exposed amphibians). In assigning these criteria, I did not critique the authors' statistics or interpretation, but accepted the narrative conclusion from their analyses.

I considered five potential factors that may contribute to positive or negative findings: (1) species, (2) study type, (3) study design, (4) principal authors, and (5) financial sponsorship. First, I asked whether the effect was restricted to certain species (i.e., whether studies with negative findings focused on species that do not respond to atrazine). Second, I considered study type, asking whether the effect was potentially a laboratory artifact or was restricted to field observations. When considering the third factor, study design, I did not examine experimental conditions in detail, but simply asked whether authors examined the gonads of atrazine-treated animals for comparison with animals that were unexposed to atrazine. If more than 50 percent of the test animals and controls died, or if control animals were exposed to atrazine equal to or in excess of the levels to which experimental animals were exposed, the design was considered inappropriate. I believed these were the minimum acceptable criteria for study design: The animals must live in

Table 1. Studies examining the effects of atrazine on the gonads of amphibians.

Reference	Recurring authors[a]	Sponsors (listed in acknowledgments)	Species	Study type[b]	Study design[c]	Conclusion (Does atrazine affect gonads?)[d]
Reeder et al. 1998	None	Midwest Society of Toxicology, John G. Shedd Aquarium	*Acris crepitans*	Field	Appropriate	Yes
Parshley 2000	None	Syngenta Crop Protection (previously Novartis Crop Protection)	*Xenopus laevis*	Laboratory	Appropriate	No
Tavera-Mendoza et al. 2002	None	Toxic Substance Research Initiative, Canada	*X. laevis*	Laboratory	Appropriate	Yes
Hayes et al. 2002a	Tyrone B. Hayes, Aaron Vonk	National Science Foundation	*X. laevis*	Laboratory	Appropriate	Yes
Hayes et al. 2002b, 2000c (laboratory studies)	Hayes, Vonk	W. Alton Jones Foundation, World Wildlife Fund, Homeland Foundation, Rose Foundation, Howard Hughes Medical Institute	*Rana pipiens*	Laboratory	Appropriate	Yes
Hayes et al. 2002b, 2000c (field studies)	Hayes, Vonk	W. Alton Jones Foundation, World Wildlife Fund, Homeland Foundation, Rose Foundation, Howard Hughes Medical Institute	*R. pipiens*	Field	Appropriate	Yes
Carr et al. 2003	Ecorisk, Inc. (John Giesy, Keith Solomon, Ernest Smith, James Carr, Ronald Kendall, Glen Van Der Kraak)	Syngenta Crop Protection	*X. laevis*	Laboratory	Inappropriate	No
Hecker 2003 (field study)	Ecorisk, Inc. (Giesy, Solomon, Smith, Carr, Kendall, Van der Kraak)	Syngenta Crop Protection	*X. laevis*	Field	Inappropriate	No
Hecker 2003 (laboratory study)	Ecorisk, Inc. (Giesy, Solomon, Smith, Carr, Kendall, Van der Kraak)	Syngenta Crop Protection	*X. laevis*	Laboratory	Inappropriate	No
Hecker et al. 2003a	Ecorisk, Inc. (Giesy, Solomon, Smith, Carr, Kendall, Van der Kraak)	Syngenta Crop Protection	*Rana clamitans*	Laboratory	Inappropriate	No
Hecker et al. 2003b	Ecorisk, Inc. (Giesy, Solomon, Smith, Carr, Kendall, Van der Kraak)	Syngenta Crop Protection	*X. laevis*	Laboratory	Inappropriate	No
Smith et al. 2003a, 2003b	Ecorisk, Inc. (Giesy, Solomon, Smith, Carr, Kendall, Van der Kraak)	Syngenta Crop Protection	*X. laevis*	Field	Inappropriate	No
Smith 2003	Ecorisk, Inc. (Giesy, Solomon, Smith, Carr, Kendall, Van der Kraak)	Syngenta Crop Protection	*X. laevis*	Field	Inappropriate	No

(*continued*)

Table 1. (*continued*)

Reference	Recurring authors[a]	Sponsors (listed in acknowledgments)	Species	Study type[b]	Study design[c]	Conclusion (Does atrazine affect gonads?)[d]
Gross et al. 2003	Ecorisk, Inc. (Giesy, Solomon, Smith, Carr, Kendall, Van der Kraak)	Syngenta Crop Protection	*Bufo marinus*	Field	Inappropriate	Yes/No
Miyahara et al. 2003	None	Towa-Kagaku (private company); Ministry of the Environment; Ministry of Education, Sports, Culture, Science, and Technology, Japan	*X. laevis*	Laboratory	Appropriate	Yes

Note: Studies that reported more than one type of experiment (laboratory and field studies) are listed more than once. For experiments whose results were reported in more than one publication, each publication is listed separately.

a. Authors are listed only if their names appear on more than one of the publications listed in this table.

b. Field studies were not distinguished from mesocosm studies in which environments were manipulated.

c. Study design was deemed inappropriate if more than 50 percent of the animals died, or if control groups had atrazine levels equal to or greater than those of the experimental groups and above the threshold for effects.

d. Conclusions on whether atrazine affected the gonads (yes or no) were determined by the authors of each study. No evaluation of statistics or interpretation was conducted. One study (Sepulveda and Gross 2003) reported both positive and negative effects (Yes/No) in separate reports (McCoy et al. 2002, Gross et al. 2003), apparently with the same data.

order for the gonads to be examined, and, as Fox (1991) wrote regarding criteria for evaluating the strength of associations in cause-and-effect analyses, the strength of the association depends on "the ratio on one side of the incidence of a disorder in the population exposed to the suspect causal factor, and on the other side, the incidence of the disorder in a comparable population *not* exposed to the suspect factor" (p. 367; emphasis added). Fourth, I considered the principal authors: Were the studies conducted by truly independent laboratories? Finally, I considered the question of financial sponsorship, that is, whether positive or negative outcomes were associated with particular financial sponsors.

I conducted a Fisher's exact test of independence to determine which of the five factors were true predictors (non-independent) of the outcome ("no effect" or "effect") on the gonads. This path analysis was very revealing. Neither the species ($P > 0.05$) nor the study type (whether the study was laboratory-or field-based; $P > 0.05$) had any effect on the outcome of the studies. Positive effects were identified in four species (from four different families), both negative and positive effects were produced in at least one species (*X. laevis*), and negative and positive effects were found in both field and laboratory studies. By contrast, financial sponsorship was a very strong predictor ($P < 0.001$): Funding sources varied for positive studies (including funding from governmental agencies from three countries—the United States, Canada, and Japan—and from multiple private companies, foundations, and agencies), whereas 100 percent of the negative studies were funded by Syngenta. Authorship ($P < 0.001$), and study design ($P < 0.001$) were also very important. Most revealing, the single negative study that did not suffer from high mortality or contaminated controls was conducted by my laboratory under the auspices of the Ecorisk panel and funded by Syngenta. The findings of this study were "reinterpreted" by Syngenta (Hayes et al. 2002a): Effects were reported as "alleged" and the report claimed that "no convincing evidence" was produced by the study (Parshley 2000) even though the data were similar to data later published by my laboratory (Hayes et al. 2002a). Even with this study, authorship and study design are very significant ($P < 0.001$). Five laboratories showed positive effects of atrazine on amphibian gonads, whereas studies with negative findings involved identical investigators (Ecorisk panel members Carr, Giesy, Ronald Kendall, Ernest Smith, Keith Solomon, and Glen Van Der Kraak) 100 percent of the time. In addition, all of the remaining negative studies suffered from high mortality, contaminated controls, or both, whereas none of the positive studies reported such problems in their design. In fact, these independent variables were highly correlated: All of the negative studies represented Syngenta-funded studies conducted by Ecorisk, and all except one had high mortality or contaminated controls ($P < 0.001$; Fisher's exact test of independence; figure 9).

Even more revealing, in a study in *Bufo marinus* in Florida, McCoy and colleagues (2002) reported that males in areas where atrazine was used displayed coloration typical of

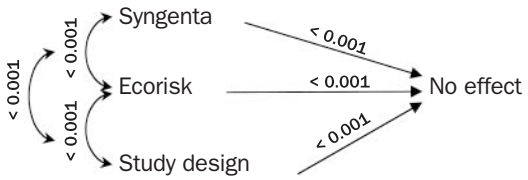

Figure 9. Path analysis showing the dependence of a negative outcome of studies of atrazine effects on amphibians ("no effect") on three predictors: sponsorship, authorship, and study design. Numbers indicate* P *values as determined by Fisher's exact test of independence. The three predictors were also not independent and were highly correlated, as indicated. The other two potential predictors, species and type of study (laboratory versus field), were not statistically significant (P > 0.05).

females and had high plasma vitellogenin levels. They also noted gonadal abnormalities, including developing (and even vitellogenic) oocytes in the Bidder's organ, and variable plasma estrogen in males (McCoy et al. 2002). In 2003, senior author Timothy Gross (a US Geological Survey employee) was quoted as saying that this study "lends credence to University of Berkeley endocrinologist Tyrone Hayes's hypothesis that atrazine is affecting sexual development of amphibians" (Renner 2002). According to Rebecca Renner's (2002) report, Gross "added that the findings are consistent with the previous work of both Hayes and Texas Tech experimental toxicologist James Carr, [and] 'Carr finds an effect at atrazine concentrations that are similar to what we see in the field and to what we think the toads are exposed.'" By 2004, now joined by coauthors from the Ecorisk panel (Carr, Giesy, Kendall, Smith, and Van Der Kraak, but with McCoy absent) and with funding from Syngenta, this same investigator (and apparently with the same data) reported that atrazine had no consistent effects on sex differentiation in this species (Gross et al. 2003).

In addition to the relationships revealed in this path analysis, the influence of the sponsor (Syngenta) and confusion generated by the Ecorisk panel are apparent even in the conflicting statements issued by one of the Ecorisk panel's own members. In 2000, Carr stated in a public press release, "We have been unable to reproduce the low-concentration effects of atrazine on amphibians reported elsewhere in the scientific literature." However, in 2004, Carr reported, "I don't think it [Carr's data] contradicts Hayes" (Blumenstyk 2003, p. A28). In the same interview, according to the *Chronicle of Higher Education,* Carr indicated that his "research speaks for itself, and that he is not responsible for how Syngenta chooses to characterize it" (Blumenstyk 2003). Further, the Syngenta-funded Ecorisk panel presented five studies at the atrazine symposium at the annual meeting of the Society for Environmental Toxicology and Chemistry in 2004, claiming to refute the findings that atrazine interferes with sex differentiation in frogs (Du Preez 2003, Gross et al. 2003, Hecker 2003, Murphy 2003, Smith 2003). Their conclusions did not reflect the sentiments expressed by Carr (a coauthor on these studies) in publicly available EPA documents regarding the sum of the work by the Syngenta-funded Ecorisk panel: "The important issue is for everyone involved to come to grips with (and stop minimizing) the fact that independent laboratories have demonstrated an effect of atrazine on gonadal differentiation in frogs. There is no denying this" (Carr 2003, p. A7).

References cited

Ankley G, Johnson RD, Detenbeck NE, Bradbury SP, Toth G, Folmar LC. 1997. Development of a research strategy for assessing the ecological risk of endocrine disruptors. Review of Toxicology and Applied Pharmacology, B: Environmental Toxicology 1: 231–267.

Aspelin AL. 1994. Pesticide Industry Sales and Usage: 1992 and 1993 Market Estimates. Washington (DC): US Environmental Protection Agency, Office of Pesticide Programs, Biological and Economic Analysis Division. Economic Analysis Branch Report no. 733-K-94-001.

Babic-Gojmerac T, Kniewald Z, Kniewald J. 1989. Testosterone metabolism in neuroendocrine organs in male rats under atrazine and deethylatrazine influence. Journal of Steroid Biochemistry 33: 141–146.

Bisson M, Hontela A. 2002. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed in vitro. Toxicology and Applied Pharmacology 180: 110–117.

Blumenstyk G. 2003. The price of research: A Berkeley scientist says a corporate sponsor tried to bury his unwelcome findings and then buy his silence. Chronicle of Higher Education. 31 October 2003, pp. A26–A29.

Cabanes A, Wang M, Gustafsson J-A, Hilakivi-Clarke L. 2004. BRCA1 effect on estrogen receptor (ER) alpha and ER beta is ligand dependent. Abstract of paper presented at the Experimental Biology 2004 meeting; 17–21 April 2004, Washington, DC. FASEB Journal 18: abstract #369.1. (10 November 2004; *http://select.biosis.org/faseb/*)

Carr J. 2003. Environmental Protection Agency, FOIA Request Identification Number HQ-RIN-0045-04.

Carr JA, Solomon KR. 2003. Is atrazine causing frog deformities at very low doses? SETAC Globe 4: 30–32.

Carr J, et al. 2003. Response of larval *Xenopus laevis* to atrazine: Assessment of growth, metamorphosis, and gonadal and laryngeal morphology. Environmental Toxicology and Chemistry 22: 396–405.

Christensen MJ, Lephart EJ. 2004. Effects of high phytoestrogen consumption on steroid hormone metabolism in Long-Evans male rats. Abstract of paper presented at the Experimental Biology 2004 meeting; 17–21 April 2004, Washington, DC. FASEB Journal 18: abstract #360.8. (10 November 2004; *http://select.biosis.org/faseb/*)

Coady K, et al. 2004. Effects of atrazine on metamorphosis, growth, and gonadal development in the green frog (*Rana clamitans*). Journal of Toxicology and Environmental Health, A 67: 941–957.

Colborn T. 1994. The wildlife/human connection: Modernizing risk decisions. Environmental Health Perspectives 102: 55–59.

Cooper RL, Stoker TE, McElroy WK. 1999. Atrazine (ATR) disrupts hypothalamic catecholamines and pituitary function. Toxicologist 42: 60–66.

Cooper RL, Stoker TE, Tyrey L, Goldman JM, McElroy WK. 2000. Atrazine disrupts the hypothalamic control of pituitary–ovarian function. Toxicological Sciences 53: 297–307.

Cummings A, Rhodes B, Cooper R. 2000. Effect of atrazine on implantation and early pregnancy in 4 strains of rats. Toxicological Sciences 58: 135–143.

Daxenberger A. 2002. Pollutants with androgen-disrupting potency. European Journal of Lipid Science and Technology 104: 124–130.

Desaulniers D, Leingartner K, Musicki B, Yagminas A, Xiao G-H, Cole J, Marro L, Charbonneau M, Tsang B. 2003. Effects of postnatal exposure to mixtures of non-ortho-PCBs, PCDDs, and PCDFs in prepubertal female rats. Toxicological Sciences 75: 468–480.

Diel P, Schmidt S, Vollmer G, Janning P, Upmeier A, Michna H, Bolt H, Degen G. 2004. Comparative responses of three rat strains (DA/Han, Sprague-Dawley and Wistar) to treatment with environmental estrogens. Archives of Toxicology 78: 183–193.

Du Preez LH. 2003. Gonadal responses of *Xenopus laevis* larvae exposed to atrazine in microcosms. Paper presented at the 24th Annual Meeting in North America, Society of Environmental Toxicology and Chemistry; 9–13 November 2003, Austin, Texas.

Eldridge JC, Fleenore-Heyser DG, Extron PC, Wetzel LT, Breckenridge CB, Gillis JH, Luempert LG III, Stevens JT. 1994a. Short-term effects of chlorotriazines on estrus in female Sprague-Dawley and Fischer 344 rats. Journal of Toxicology and Environmental Health 43: 155–167.

Eldridge JC, Tennant MK, Wetzel LC, Breckenridge CB, Stevens JT. 1994b. Factors affecting mammary tumor incidence in chlorotriazine-treated female rats: Hormonal properties, dosage, and animal strain. Environmental Health Perspectives 102: 29–36.

Fenner-Crisp PA. 1997. Endocrine disruptor risk characterization: An EPA perspective. Regulatory Toxicology and Pharmacology 26: 70–73.

Fox G. 1991. Practical causal inference for epidemiologists. Journal of Toxicology and Environmental Health 33: 359–373.

Friedmann A. 2002a. Atrazine inhibition of testosterone production in rat males following peripubertal exposure. Reproductive Toxicology 16: 275–279.

———. 2002b. Atrazine inhibition of testosterone production in rat males following peripubertal exposure. Reproductive Toxicology 16: 275–279.

Gadamski G, Ciarka D, Gressel J, Gawronski SW. 2003. Negative cross-resistance in triazine-resistant biotypes of *Echinochloa crus-galli* and *Conyza canadensis.* Weed Science 48: 176–180.

Gebhard R, Van Der Voort H, Schuts W, Schoonen W. 1997. 11,21-bisphenyl-19-norpregnane derivatives are selective antiglucocorticoids. Bioorganic and Medicinal Chemistry Letters 7: 2229–2234.

Green D. 2003. The ecology of extinction: Population fluctuation and decline in amphibians. Biological Conservation 111: 331–343.

Gross TS, Smith EE, Wiebe E, Sepulveda MS, Carr J, Du Preez LJ, Kendall RJ, Solomon K, Van Der Kraak G. 2003. An evaluation of gonadal anomalies and agrichemical exposures for the cane toad (*Bufo marinus*) in south Florida. Paper presented at the 24th Annual Meeting in North America, Society of Environmental Toxicology and Chemistry; 9–13 November 2003, Austin, Texas.

Harvey PW, Johnson I. 2002. Approaches to the assessment of toxicity data with endpoints related to endocrine disruption. Journal of Applied Toxicology 22: 241–247.

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A. 2002a. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences 99: 5476–5480.

Hayes TB, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. 2002b. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): Laboratory and field evidence. Environmental Health Perspectives 111: 568–575.

———. 2002c. Feminization of male frogs in the wild. Nature 419: 895–896.

Heap I. 1997. The occurrence of herbicide-resistant weeds worldwide. Pesticide Science 51: 235–245.

Hecker MJ. 2003. Effects of atrazine on adult *Xenopus laevis* in the wild and laboratory: No evidence for an aromatase-based mechanism. Paper presented at the 24th Annual Meeting in North America, Society of Environmental Toxicology and Chemistry; 9–13 November 2003, Austin, Texas.

Hecker MJ, Coady KK, Villeneuve DL, Murphy MB, Jones PD, Giesy JP. 2003a. A Pilot Study of Response of Larval *Rana clamitans* to Atrazine Exposure: Assessment of Metamorphosis and Gonadal and Laryngeal Morphology and Selected Hormones and Enzyme Activities. Ferndale (WA): Ecorisk. Interim Report MSU-03.

———. 2003b. Response of *Xenopus laevis* to Atrazine Exposure: Assessment of the Mechanism of Action of Atrazine. Ferndale (WA): Ecorisk. Interim Report MSU-04.

Hecker MJ, Giesy JP, Jones P, Jooste AM, Carr J, Solomon KR, Smith EE, Van Der Kraak G, Kendall RJ, Du Preez LH. 2004. Plasma sex steroid concentrations and gonadal aromatase activities in African clawed frogs (*Xenopus laevis*) from South Africa. Environmental Toxicology and Chemistry 23: 1996–2007.

Horiguchi T, Li Z, Uno S, Shimizu M, Shiraishi H, Morita M, Thompson J, Levings C. 2004. Contamination of organotin compounds and imposex in molluscs from Vancouver, Canada. Marine Environmental Research 57: 75–88.

Hosmer AJ. 2003. Frogs and atrazine. SETAC Globe 4: 37.

Kadereit JW. 1984. The origin of *Senecio vulgaris* (Asteraceae). Plant Systematics and Evolution 145: 135–153.

Kendall RJ, Bruce RL, Carr JA, Du Preez L, Giesy J, Gross T, Smith EE, Solomon K, Van Der Kraak G. 2002. Frog research on atrazine casts doubt on earlier studies. (10 November 2004; *www.farmassist.com/prod/herbicide/atrazine/index.asp?nav=Ecorisk*)

Kirigaya A, Sato T, Hayashi S. 2003. Developmental effects of ethynyl estradiol (EE2) on reproductive organs in female mice. Zoological Science (Tokyo) 20: 1608.

Kniewald J, Mildner P, Kniewald Z. 1979. Effects of s-triazine herbicides on hormone-receptor complex formation, 5 alpha-reductase and 3 alpha-hydroxysteroid dehydrogenase activity at the anterior pituitary level. Journal of Steroid Biochemistry 11: 833–838.

———. 1980. Effects of s-triazine herbicides on 5 alpha-dihydrotestosterone receptor complex formation in the hypothalamus and ventral prostate. Pages 159–169 in Genazzani F, DiCarlo F, Mainwaring WIP, eds. Pharmacological Modulation of Steroid Action. New York: Raven Press.

Kniewald J, Osredecki V, Gojmerac T, Zechner V, Kniewald Z. 1995. Effect of s-triazine compounds on testosterone metabolism in the rat prostate. Journal of Applied Toxicology 15: 215–218.

Kniewald J, Jakominic M, Tomljenovic A, Simic B, Romac P, Vranesic D, Kniewald Z. 2000. Disorders of male rat reproductive tract under the influence of atrazine. Journal of Applied Toxicology 20: 61–68.

Leanos-Castaneda O, Van Der Kraak G, Lister A, Sima-Alvarez R, Gold-Bouchot G. 2002. O,p'-DDT induction of vitellogenesis and its inhibition by tamoxifen in Nile tilapia (*Oreochromis niloticus*). Marine Environmental Research 54: 703–707.

Lundholm CE, Bartonek M. 1991. A study of the effects of p,p'-DDE and other related chlorinated hydrocarbons on inhibition of platelet aggregation. Archives of Toxicology 65: 570–574.

Marshall D, Rajkumar A. 2003. Imposex in the indigenous *Nassarius kraussianus* (Mollusca: Neogastropoda) from South African harbours. Marine Pollution Bulletin 46: 1150–1155.

Masutomi N, Shibutani M, Takagi H, Uneyama C, Lee K-Y, Hirose M. 2004. Alteration of pituitary hormone-immunoreactive cell populations in rat offspring after maternal dietary exposure to endocrine-active chemicals. Archives of Toxicology 78: 232–240.

McCoy KA, Sepulveda MS, Gross TS. 2002. Atrazine exposure and reproductive system abnormalities in field collected *Bufo marinus.* Paper presented at the 23rd Annual Meeting in North America, Society of Environmental Toxicology and Chemistry; 16–20 November 2002, Salt Lake City, Utah.

Miyahara M, Oka T, Mitsui N, Sagoe C, Kashiwagi A, Shinkai T, Sone K, Tooi O, Iguchi T 2003. Evaluation of atrazine on *Xenopus laevis* in a partial life test. Paper prepared for the Sixth Annual Meeting of the Japan Society of Endocrine Disrupters Research; 2–3 December 2003, Sendai, Japan. Abstract #PB-57.

[MRC IEH] Medical Research Council Institute for Environment and Health Assessment. 1999. IEH Assessment on the Ecological Significance of Endocrine Disruption: Effects on Reproductive Function and Consequences for Natural Populations. Leicester (United Kingdom): MRC IEH.

Murphy M. 2003. Plasma testosterone and estradiol concentrations, aromatase activities and gonad histology of ranids in Michigan. Paper presented at the 24th Annual Meeting in North America, Society of Environmental Toxicology and Chemistry; 9–13 November 2003, Austin, Texas.

Naciff J, Overmann G, Torontali S, Carr G, Tiesman J, Richardson B, Daston G. 2003. Gene expression profile induced by 17 alpha-ethynyl estradiol in the prepubertal female reproductive system of the rat. Toxicological Sciences 72: 314–330.

Narotsky M, Best DS, Guidici DL, Cooper RL. 2001. Strain comparisons of atrazine-induced pregnancy loss in the rat. Reproductive Toxicology 15: 61–69.

Noriega N, Hayes TB. 2000. DDT congener effects on secondary sex coloration in the reed frog *Hyperolius argus:* A partial evaluation of the *Hyperolius argus* endocrine screen. Comparative Biochemistry and Physiology, B26: 231–237.

Parshley T. 2000. Report of an Alleged Adverse Effect from Atrazine: Atrazine technical, EPA. Reg. no. 100-529. Report to the EPA.

Reeder A, et al. 1998. Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). Environmental Health Perspectives 106: 261–266.

Reitsema T, Field S, Spickett J. 2003. Surveying imposex in the coastal waters of Perth, Western Australia, to monitor trends in TBT contamination. Australian Journal of Ecotoxicology 9: 87–92.

Renner R. 2002. More evidence that herbicides feminize amphibians. Environmental Science and Technology 37: 46A.

Robertson J, Watson J, Kelley D. 1994. Androgen directs sexual differentiation of laryngeal innervation in developing *Xenopus laevis.* Journal of Neurobiology 25: 1625–1636.

Sanderson JT, Seinen W, Giesy JP, van den Berg M. 2000. 2-chloro-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: A novel mechanism for estrogenicity? Toxicological Sciences 54: 121–127.

Sanderson JT, Letcher RJ, Heneweer M, Giesy JP, van den Berg M. 2001. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environmental Health Perspectives 109: 1027–1031.

Schmidt BR. 2003. Count data, detection probabilities, and the demography, dynamics, distribution, and decline of amphibians. Comptes Rendus Biologies 326: S119–S124.

Sepulveda MS, Gross TS. 2003. Characterization of Atrazine Exposures and Potential Effects in Florida Ecosystems Dominated by Sugarcane Agriculture: A Reconnaissance Survey of Amphibians in South Florida for the Assessment of Potential Atrazine Effects. Ferndale (WA): Ecorisk. Technical Report no. UFL-02.

Simic B, Kniewald Z, Davies J, Kniewald J. 1991. Reversibility of the inhibitory effect of atrazine and lindane on cytosol 5 alpha-dihydrotestosterone receptor complex formation in rat prostate. Bulletin of Environmental Contamination and Toxicology 46: 92–99.

Simic B, Kniewald J, Kniewald Z. 1994. Effect of atrazine on reproductive performance in the rat. Journal of Applied Toxicology 14: 401–404.

Smith EE. 2003. Stereology: Assessment of gonadal function in *Xenopus laevis* from corn-growing areas of South Africa. Paper presented at the 24th Annual Meeting in North America, Society of Environmental Toxicology and Chemistry; 9–13 November 2003, Austin, Texas.

Smith EE, Du Preez LH, Solomon KR. 2003a. Field Exposure of *Xenopus laevis* to Atrazine and Other Triazines in South Africa: Exposure Characterization and Assessment of Laryngeal and Gonadal Responses. Ferndale (WA): Ecorisk. Technical Report no. SA-01A.

———. 2003b. Gonadal and Laryngeal Responses to Field Exposure of *Xenopus laevis* to Atrazine in Areas of Corn Production in South Africa. Ferndale (WA): Ecorisk. Final Report no. SA-01C.

Steeger TM, Tietge JE, Irene S, Frankenberry MJ. 2003a. Data Evaluation Report on a Pilot Study of Larval *R. clamitans* Response to Atrazine Exposure in Terms of Metamorphosis, Gonadal and Laryngeal Morphology and Selected Hormonal and Enzymatic Activities. EPA MRID no. 458677-03. (10 November 2004; *www.epa.gov/oscpmont/sap/2003/june/dataevaluationreports.htm*)

———. 2003b. Data Evaluation Report on Field Exposure of *Xenopus laevis* to Atrazine and Other Triazines in South Africa: Feasibility Study for Site Characterization and Assessment of Laryngeal and Gonadal Responses. EPA MRID no. 458677-09. (10 November 2004; *www.epa.gov/oscpmont/sap/2003/june/dataevaluationreports.htm*)

———. 2003c. Data Evaluation Report on Gonadal and Laryngeal Responses to Field Exposure of *Xenopus laevis* to Atrazine in Areas of Corn Production in South Africa. EPA MRID no. 458677-10. (10 November 2004; *www.epa.gov/oscpmont/sap/2003/june/dataevaluationreports.htm*)

———. 2003d. Data Evaluation Report on the Reconnaissance Survey of South Florida Amphibians for the Assessment of Potential Atrazine Effects. EPA MRID no. 458677-06. (10 November 2004; *www.epa.gov/oscpmont/sap/2003/june/dataevaluationreports.htm*)

———. 2003e. Data Evaluation Report on Response of *Xenopus laevis* to Atrazine Exposure: Assessment of Mechanism of Action of Atrazine. EPA MRID no. 458677-04. (10 November 2004; *www.epa.gov/oscpmont/sap/2003/june/dataevaluationreports.htm*)

Stevens JT, Breckenridge CB, Wetzel LT, Gillis JH, Luempert LG, Eldridge JC. 1994. Hypothesis for mammary tumorigenesis in Sprague-Dawley rats exposed to certain triazine herbicides. Journal of Toxicology and Environmental Health 43: 139–154.

Stickle W. 2003. Long-term trends in imposex in six populations of *Stramonita haemastoma.* Bulletin of Marine Science 72: 685–694.

Storfer A. 2003. Amphibian declines: Future directions. Diversity and Distributions 9: 151–163.

Takagi H, Shibutani M, Takahashi N, Mitsumori K, Hirose M. 2004. Lack of maternal dietary exposure effects of bisphenol A and nonylphenol during the critical period for brain sexual differentiation on the reproductive/endocrine systems in later life. Archives of Toxicology 78: 97–105.

Tavera-Mendoza L, Ruby S, Brousseau P, Fournier M, Cyr D, Marcogliese D. 2002. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. Environmental Toxicology and Chemistry 21: 527–531.

Tennant MK, Hill DS, Eldridge JC, Wetzel LT, Breckenridge CB, Stevens JT. 1994. Chloro-s-triazine antagonism of estrogen action: Limited interaction with estrogen receptor binding. Journal of Toxicology and Environmental Health 43: 197–211.

Terasaki M, Nomachi M, Edmonds J, Morita M. 2004. Impurities in industrial grade 4,4'-isopropylidene diphenol (bisphenol A): Possible implications for estrogenic activity. Chemosphere 55: 927–931.

Tobias M, Marin ML, Kelley D. 1991. Development of functional sex differences in the larynx of *Xenopus laevis.* Developmental Biology 147: 251–259.

———. 1993. The roles of sex, innervation, and androgen in laryngeal muscle of *Xenopus laevis.* Journal of Neuroscience 13: 324–333.

Tollefsen K-E, Mathisen R, Stenersen J. 2002. Estrogen mimics bind with similar affinity and specificity to the hepatic estrogen receptor in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). General and Comparative Endocrinology 126: 14–22.

Trentacoste S, Friedmann A, Youker R, Breckenridge C, Zirkin B. 2001. Atrazine effects on testosterone levels and androgen-dependent reproductive organs in peripubertal male rats. Journal of Andrology 22: 142–148.

Vaya J, Tamir S. 2004. The relation between the chemical structure of flavonoids and their estrogen-like activities. Current Medicinal Chemistry 11: 1333–1343.

Wetzel LT, Luempert LG, Breckenridge CB, Tisdel MO, Stevens JT, Thakur AK, Extrom PJ, Eldridge JC. 1994. Chronic effects of atrazine on estrus and mammary gland formation in female Sprague-Dawley and Fischer-344 rats. Journal of Toxicology and Environmental Health 43: 169–182.