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A Qualitative Meta-analysis Reveals Consistent Effects of Atrazine on Freshwater Fish and Amphibians

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Abbreviations:

EEC	expected environmental concentration
LOEC	lowest observable effect concentrations
TOF	testicular ovarian follicle
USDA	United States Department of Agriculture

Outline of Section Headers

OBJECTIVE: The biological effects of the herbicide atrazine on freshwater vertebrates are highly controversial. In an effort to resolve the controversy, we conducted a qualitative meta-analysis on the effects of ecologically relevant atrazine concentrations on amphibian and fish survival, behavior, metamorphic traits, infections, and immune, endocrine, and reproductive systems.

DATA SOURCES: We used published, peer-reviewed research and applied strict quality criteria for inclusion of studies in the meta-analysis.

DATA SYNTHESIS: We found little evidence that atrazine consistently caused direct mortality of fish or amphibians, but found evidence that it can have indirect and sub-lethal effects. The relationship between atrazine concentration and timing of amphibian metamorphosis was regularly non-monotonic, indicating that atrazine can both accelerate and delay metamorphosis. Atrazine reduced size at or near metamorphosis in 19 of 19 studies. Atrazine elevated amphibian and fish activity in 12 of 14 studies, reduced anti-predator behaviors in six of seven studies, and reduced olfactory abilities for fish but not for amphibians. Atrazine was associated with a reduction in 35 of 43 immune function endpoints and with an increase in 13 of 16 infection endpoints. Atrazine altered at least one aspect of gonadal morphology in eight of 10 studies, and consistently affected gonadal function, altering spermatogenesis in two of two studies and sex hormone concentrations in six of seven studies. Atrazine did not affect vitellogenin in five studies and only increased aromatase in one of six studies. Effects of atrazine on fish and amphibian reproductive success, sex ratios, gene frequencies, populations, and communities remain uncertain.

CONCLUSIONS: Although there is much left to learn about the effects of atrazine, we identified several consistent effects of atrazine that must be weighed against any of its benefits and the costs and benefits of alternatives to atrazine use.

INTRODUCTION

The herbicide atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) is the second most commonly used pesticide in the United States (Kiely et al. 2004), and perhaps the world (Solomon et al. 1996; van Dijk and Guicherit 1999). It is a photosynthesis inhibitor used to control certain annual broadleaf weeds, predominantly in corn but also in sorghum, sugarcane, and other crops and landscaping. The environmental risk posed by atrazine to aquatic systems is presently being re-evaluated by the US Environmental Protection Agency (USEPA: USEPA 2003, 2007). One of the challenges in evaluating the safety of atrazine has been that its biological effects are highly controversial, and much of the debate in the literature has been targeted at its effects on freshwater vertebrates (Hayes 2004; Renner 2004).

There have been four reviews on the biological effects of atrazine, all of which were funded by the corporation that produced or produces this chemical (Giddings et al. 2005; Huber 1993; Solomon et al. 1996; Solomon et al. 2008). However, none of the past reviews used a meta-analytical approach to identify generalities in responses to atrazine exposure. Meta-analysis, as paraphrased from the USEPA, is the systematic analysis of studies examining similar endpoints to draw general conclusions, develop support for hypotheses, and/or produce an estimate of overall effects. This sort of weight-of-evidence approach would provide directional hypotheses for future work on atrazine. Furthermore, it would offer invaluable information to regulatory agencies on general and expected impacts of atrazine on freshwater vertebrates that might help resolve much of the controversy surrounding atrazine. Given the lack of a meta-analytical assessment and the potential importance of any atrazine effects, we set out to conduct an objective, qualitative meta-analysis on the effects of atrazine on amphibian and fish survival, behavior, metamorphic traits, and immune, endocrine, and reproductive systems.

ATRAZINE PERSISTENCE, TRANSPORT, AND EXPOSURE

To place the results of this meta-analysis within an ecological context and to evaluate the relevance of studied atrazine concentrations and exposure regimes, we briefly discuss the fate, transport, and field concentrations of atrazine. Atrazine is persistent relative to most current-use pesticides. Ciba-Giegy Corporation (1994), the previous atrazine producer, reported no detectable change in atrazine concentration after 30 d in hydrolysis studies conducted at pHs between 5 and 7, and an aqueous photolysis half-life of 335 days under natural light and a neutral pH. Half-lives from field and mesocosms studies are variable because degradation can depend on various environmental conditions. Nevertheless, several field and mesocosm studies report half-lives over three months (e.g. de Noyelles et al. 1989; Klaassen and Kadoum 1979).

Atrazine is also relatively mobile, regularly entering water bodies through run-off, with concentrations in surface waters often peaking after rains. Several researchers have suggested that atrazine can be transported 1000 km aerially (see van Dijk and Guicherit 1999). Indeed, atrazine has regularly been found in surface waters and precipitation great distances from where it is used, such as above the Arctic circle, albeit at low concentrations (van Dijk and Guicherit 1999).

Wet deposition of atrazine might also be important in some areas. In a review on atmospheric dispersion of current-use pesticides, van Dijk and Guicherit (1999) report more studies detecting atrazine in rain or air (from European and US sites) than any other current-use pesticide. The maximum reported wet deposition of atrazine is 154 µg/L from Iowa, USA precipitation (Hatfield et al. 1996). Wet deposition above 1 µg/L has been reported regularly in North America and Europe between 1980 and the early 1990s (reviewed by van Dijk and Guicherit 1999). As a reference point, the maximum contaminant level for drinking water set by the USEPA is 3 µg/L of atrazine (USEPA 2002).

Surface water is likely the primary source of atrazine exposure for freshwater vertebrates. Data on atrazine concentrations in surface water, however, are more abundant for lotic (streams and rivers) than lentic (lakes, ponds, wetlands, ditches) systems (Solomon et al. 2008), primarily because of the extensive stream monitoring conducted by the US Geological Survey NAWQA project and Syngenta Crop Protection, Inc. (USEPA 2007). In lentic systems, water is not replenished like it is in lotic systems and chemicals can concentrate as lentic systems dry. Maximum reported concentrations in lentic systems are often between 2.5 to 10 times higher than maximum concentrations in lotic systems (Baker and Laflen 1979; Edwards et al. 1997; Evans and Duseja 1973; Frank et al. 1990; Kadoum and Mock 1978; Kolpin et al. 1997). Additionally, many amphibians develop in ephemeral agricultural ponds that might receive and concentrate atrazine (Knutson et al. 2004).

Given the limited data on atrazine concentrations in lentic systems, the expected (or estimated) environmental concentration (EEC) is a reasonable alternative for estimating concentrations to which aquatic organisms are likely to be exposed. The USEPA GENEEC v2 software calculates standardized EECs that are used by the USEPA for Tier 1 chemical risk screening. EECs are important because chemical registration decisions entail comparing lowest observable effect concentrations (LOEC) to EECs to determine whether higher level modeling is warranted. Hence, effects of a chemical near or below the EEC can affect the decision to approve its use.

For present atrazine application rates, EECs based on GENEEC v2 software are typically near 100 µg/L but can be higher for some crops. However, the recommended application rates are now (~2lbs active ingredient/acre) two to four times less than they were in the early 1990s (~8 lbs active ingredient/acre). Hence, at the time of atrazine registration, LOECs near or below 500 µg/L, a feasible EEC at the time, might have triggered Tier 2 testing and might have raised concerns about the safety of atrazine that could have compromised its registration. Given both past and present day conditions, the lack of thorough data on atrazine concentrations in lentic systems, and the common use of agricultural ponds, ditches, and wetlands by amphibians and fish, we suggest that concentrations near or below historical EECs (≤500 µg/L) are ecologically relevant when considering the findings of this meta-analysis. This is arguably conservative given that atrazine concentrations have been regularly recorded in agricultural ponds and ditches above 500 µg/L (Baker and Laflen 1979; Edwards et al. 1997; Evans and Duseja 1973; Frank et al. 1990; Kadoum and Mock 1978; Kolpin et al. 1997).

METHODS

We selected studies for this meta-analysis by starting with those cited by Solomon et al. (2008), the most recent review of atrazine effects on amphibians and fish. We then supplemented these studies with a Web of Science search to identify studies that might have been missed by Solomon et al. (2008). The search terms were “atrazine” combined with either “amphibian*” or “fish*”.

Selection criteria for inclusion of studies in meta-analyses can affect the conclusions that are drawn (Englund et al. 1999). Hence, we excluded studies from this meta-analysis that had substantial contamination in control treatments or reference sites (unless a regression approach was taken to analyze the data), no presentation of statistics and within-group variance estimates, considerable inconsistencies that could affect the biological conclusions, spatial confounders associated with atrazine treatments, pseudoreplication, or other considerable flaws in experimental design. We evaluated whether the exclusion of these studies changed the conclusion of the meta-analysis for each endpoint (Englund et al. 1999). Out of the 15 response variables, never did including studies that did not meet our criteria alter the conclusions of our meta-analyses and in some cases they actually strengthened the conclusions. Because of this and space limitations, which studies were excluded and why, as well as the directions of effects in these studies, are only provided in Supplemental Material.

We chose to conduct a qualitative meta-analysis, where we tallied the number of studies that did and did not detect effects of atrazine ("vote-counting" method), for several reasons. We quantify the effects of atrazine on 15 response variables from over 125 studies, and vote-counting, the simplest approach to meta-analyses, made it feasible to manage this complexity. Vote-counting also facilitates identifying response variables that might warrant more sophisticated meta-analyses based on effect sizes. Finally, vote-counting was chosen because it is a conservative approach, biasing results towards detecting no overall effect (Gurevitch and Hedges 1993). Because most atrazine studies conducted analysis of variance to test for dose-responses, despite regression analyses providing much greater statistical power (Cottingham et al. 2005), we also include studies that had substantial trends for effects of atrazine with significant effects and make this lumping clear in tables and text.

RESULTS AND DISCUSSION

Effects of Atrazine on Fish and Amphibian Survival

Many researchers have evaluated the effects of atrazine on fish (reviewed by Giddings et al. 2005; Huber 1993; Solomon et al. 1996) and amphibian survival (e.g. Allran and Karasov 2000, 2001; Brodeur et al. 2009; Diana et al. 2000; Freeman and Rayburn 2005; Rohr et al. 2003, 2004; Rohr et al. 2006b). Our general conclusion from these studies are consistent with the conclusions of authors from previous atrazine reviews (Giddings et al. 2005; Huber 1993; Solomon et al. 1996; Solomon et al. 2008) – there is not consistent, published evidence that ecologically relevant concentrations of atrazine are *directly* toxic to fish or amphibians. There are, however, some important exceptions (e.g. Alvarez and Fuiman 2005; Rohr et al. 2006b; 2008c; Storrs and Kiesecker 2004). Given journal space limitations and that our conclusions are consistent with previous reviews, we did not conduct a meta-analysis on survival.

Effects of Atrazine on Fish and Amphibian Development and Growth

Background on metamorphosis

A basic understanding of four concepts about amphibian metamorphosis is necessary to interpret the effects of any chemical on time to, or size at, metamorphosis. First, amphibians must reach a minimum size before they can metamorphose (Wilbur and Collins 1973). Second, once they reach this size, they can accelerate development and metamorphose earlier if they are in a "stressful" environment or metamorphose later if they are in a "good" environment (Wilbur and Collins 1973). Last, metamorphosis is predominantly controlled by corticosterone and thyroid hormones (Larson et al. 1998), thus endocrine system disruption can lead to inappropriately timed metamorphosis.

These important facts have profound implications for understanding the effects of pollution on metamorphic traits. For example, imagine that an amphibian shunts energy away from growth to detoxify a chemical and, as a result, reaches the minimum size for metamorphosis five days later than amphibians not exposed to the chemical. Once this amphibian reaches the minimum size for metamorphosis, it might accelerate its developmental rate and metamorphose five days earlier to get out of the “stressful” chemical environment. In this example, there is no net effect of the chemical on time to metamorphosis despite it inarguably having considerable effects on energy use, growth, and developmental (Larson et al. 1998). A single chemical could delay, accelerate, or have no effect on timing of metamorphosis depending on chemical type and concentration.

This example was meant to highlight four points. First, a lack of an effect of a chemical on timing of metamorphosis does not mean there was no effect on developmental rate or hormones that drive metamorphosis, as Solomon et al. (2008) conclude. Second, non-monotonic dose-responses in the timing of metamorphosis are expected and are likely common. This is because there are several processes occurring (detoxification, growth, and modulation of developmental timing) that can be temporally offset and that likely have different (and potentially opposite) functional responses to the same chemical. Third, timing of metamorphosis in response to chemicals should be highly variable. This variation should not be interpreted as inconsistencies across studies (e.g. Solomon et al. 2008) because the complexity of metamorphosis is expected to induce extreme variability. Finally, unlike timing of metamorphosis, size at metamorphosis is expected to monotonically decrease with increasing chemical concentration across species and studies (controlling for time to metamorphosis). This is because energy used for detoxification is often taken away from that used for growth and development.

Effects on metamorphic traits

Our qualitative meta-analysis on the effects of atrazine on metamorphic traits is consistent with the predictions just described. Thirteen of 21 studies found significant effects of atrazine on metamorphic timing, with seven showing an increase and seven showing a decrease in time to metamorphosis, and thus, as predicted, the direction of the effect was not consistent across studies (Table 1). Seven of the 21 studies had either clear non-monotonic dose-responses or were possibly non-monotonic (Table 1). These results are consistent with the high variability and high probability of non-monotonicity expected for this endpoint.

Only two studies explicitly quantified the effects of atrazine on both thyroid hormones and timing of metamorphosis, and both showed significant non-monotonic effects (Freeman et al. 2005; Larson et al. 1998; Table 1). Further, Larson et al. (1998) revealed delays in growth and development early in life followed by accelerated development and early metamorphosis once a critical size for metamorphosis was reached. Additional studies that quantify the impacts of atrazine on thyroid hormones, corticosteroid hormones, and changes in growth and development through time are needed.

In contrast to timing of metamorphosis, size at metamorphosis shows a clear dose-dependent response to atrazine exposure (Table 1). Nineteen out of nineteen studies reported that atrazine was associated with significant reductions, or considerable trends toward reductions, in amphibian size at metamorphosis, and all of these studies reported effects at ecologically relevant concentrations based on the above criteria (Table 1). Similar growth reductions have been observed in fish (Alvarez and Fuiman 2005; McCarthy and Fuiman 2008). Atrazine consistently reduced amphibian size, which is likely to have adverse effects on amphibian populations because smaller metamorphs generally have lower terrestrial survival, lower lifetime reproduction, and compromised immune function (Carey et al. 1999, Scott 1994, Smith 1987). However, population-level effects of atrazine have not been empirically tested for in nature, and thus need to be evaluated explicitly.

Effects of Atrazine on Fish and Amphibian Behavior

Effects on locomotor activity

Twelve out of fourteen studies reported that atrazine exposure increased amphibian or fish locomotor activity over at least a portion of the concentration gradient tested (Table 2). Interestingly, four out five studies on fish, but none of the studies on amphibians, reported non-monotonic dose responses. For fish, low concentrations of atrazine stimulated hyperactivity but higher concentrations caused reductions in activity. For amphibians, hyperactivity was typically observed at the concentrations tested, but higher concentrations would likely eventually become toxic and reduce activity. All studies conducted on fish detected effects of atrazine on locomotor activity, whereas 75% of the studies on amphibians detected atrazine effects (Table 2).

The effects of atrazine on amphibian and fish locomotor activity are consistent with atrazine-induced changes in locomotor activity in mammals. Atrazine seems to cause hyperactivity in mammals by competing with receptors for the inhibitory neurotransmitter gamma aminobutyric acid, by altering monoamine turnover, and through neurotoxicity of the dopaminergic system (Das et al. 2001; Rodriguez et al. 2005). One study showed that atrazine has similar effects on the nervous system of Ranid frogs (Papaefthimiou et al. 2003), but additional studies are needed that evaluate the mechanisms responsible for atrazine-induced activity changes in fish and amphibians.

Effects on anti-predator behaviors

Six out of seven studies reported that atrazine decreased amphibian and fish behaviors associated with “predation-related” risk reduction (Table 2). Reduced predation avoidance behaviors increases predation risk, whereas increased hyperactivity (noted above) should increase encounter rates with predators (Skelly 1994). Hence, reduced risk-reduction behaviors coupled with hyperactivity is expected to increase predation. However, there are no published studies on the effects of atrazine on predator-prey relationships to which we are aware. Given that atrazine might have effects on both predators and prey, the effects of atrazine on predator-prey interactions are difficult to predict without additional studies.

Effects on olfaction

Five out of five studies reported that atrazine exposure reduced olfactory sensitivity of fish in a dose-dependent manner (Table 2). In contrast, three out of three studies on amphibians detected no effects of atrazine on olfaction at much higher concentrations than were tested on fish (Table 2). One study on amphibians stained activated olfactory neurons with agmatine and found no difference in the stimulation of olfactory neurons between atrazine-treated and control animals (Lanzel 2008).

Effects on other behaviors

One study showed that atrazine reduced amphibian water conserving behaviors which increased their rate of water loss (Rohr and Palmer 2005) (Table 2). Interestingly, both the hyperactivity and the reduced water conserving behaviors in this study occurred hundreds of days after atrazine exposure had ceased and there was no evidence that these endpoints recovered from atrazine exposure, suggesting permanent effects (Rohr and Palmer 2005). Amphibians are extremely susceptible to desiccation, and thus atrazine-induced changes in water conserving behaviors would be expected to increase mortality risk.

Effects of Atrazine on Fish and Amphibian Immunity and Infections

Effects on immunity

Our qualitative meta-analysis revealed that atrazine exposure consistently reduced immune functioning of fish and amphibians, with 16 of 18 studies finding effects at ecologically relevant concentrations. However, many of the endpoints (16/39) were from studies where atrazine was tested as part of a mixture of pesticides, and thus the effects of atrazine were not isolated (Table 3). Nevertheless, atrazine exposure, alone (22/27 endpoints) or in a pesticide mixture (13/16 endpoints), was associated with reduced immune functioning, resulting in an overall reduction in 81% (35/43) of the quantified fish and amphibian immune endpoints (including trends for a decrease; Table 3). These results are somewhat conservative because in one study multiple genes associated with immunity were significantly down-regulated (Langerveld et al. 2009), but they were counted as a single endpoint (Table 3).

Effects on infections

Similar to the effects of atrazine on amphibian and fish immunity, atrazine exposure was consistently associated with an increase in infection endpoints in fish and amphibians at ecologically relevant concentrations (Table 4). Atrazine elevated trematode, nematode, viral, and bacterial infections (Table 4). Of the studies with sufficient statistical power and without obvious confounders, 12 out of 14 of the infection endpoints increased or showed a strong trend toward increasing, indicating either more infected individuals, more infections per individual, faster maturation or greater reproduction of the parasite within the host, or greater parasite-induced host mortality (Table 4). As with immunity, these patterns should be considered with caution because many of these endpoints (6/16) came from studies where atrazine was part of a mixture of pesticides tested. Nevertheless, atrazine exposure, alone (4/7 endpoints) or in a pesticide mixture or field study (9/9 endpoints), was associated with an increase in infection endpoints (Table 4). In general, high concentrations of atrazine seem to be directly toxic to trematodes and viruses, possibly reducing infection risk for amphibians (Forson and Storfer 2006a; Koprivnikar et al. 2006, 2007; Rohr et al. 2008b), whereas more ecologically common concentrations seem to increase amphibian susceptibility, elevating infection risk (Forson and Storfer 2006b; Gendron et al. 2003; Kiesecker 2002; Rohr et al. 2008c).

Several atrazine studies only collected immunological data from animals that were also exposed to parasites, thus confounding immune parameters with parasite exposure and loads (Christin et al. 2003; Forson and Storfer 2006b; Gendron et al. 2003; Hayes et al. 2006; Kiesecker 2002; Rohr et al. 2008c). However, in every one of these studies, atrazine was associated with both reduced immune parameters and elevated parasite loads. Parasites reducing immune responses cannot explain the elevated infections associated with atrazine. Hence, the parsimonious explanation for both of these findings is that atrazine reduced immune responses which elevated infections, especially given that vertebrates typically up-regulate immunity upon infection (Raffel et al. 2006).

Despite the apparent consistency in the effects of atrazine on immunity and infections (Table 3), much remains to be learned about the effects of atrazine, and other chemicals, on parasite-host interactions (Raffel et al. 2008; Rohr et al. 2006a). For instance, we know little about how atrazine-induced changes affect population or community dynamics or most human diseases.

Effects of Atrazine on Fish and Amphibian Gonadal Morphology

General morphological endpoints

Sex differentiation is the process by which gonads develop into either testes or ovaries from an undifferentiated or bi-potential gonad (Hayes 1998). This process is distinct from reproductive maturation where the differentiated gonad becomes reproductively functional (e.g., undergoes spermatogenesis, in males). Determining if atrazine induces changes in gonadal morphology is an important step in evaluating whether it can influence sexual differentiation.

Atrazine consistently affected male gonadal morphology in fish and amphibians (Table 5). Eight of the 10 studies included in our meta-analysis report strong trends or statistically significant (six studies) alterations in at least one aspect of general gonadal morphology associated with atrazine exposure. Alterations included discontinuous and multiple testes, sexually ambiguous gonadal tissue, testicular ovarian follicles, altered gonadal somatic index (GSI- body size corrected gonadal size), expanded testicular lobules and spermatogenic tubule diameter (Table 5).

Effects on ovarian morphology are generally less obvious than those on testicular morphology and are typically dismissed without quantification. None of the three studies on fish or amphibians included in our meta-analysis found significant effects of atrazine on ovarian morphology, suggesting that atrazine induces fewer gonadal abnormalities in females than males. However, additional studies are necessary to fully evaluate the effects of atrazine on female gonadal morphology.

Testicular ovarian follicles as a natural phenomenon

Jooste et al (2005) and Solomon et al. (2008) argue that experiments with high numbers of testicular ovarian follicles (TOFs) in control *X. laevis* support the hypothesis that TOFs are normal in some *X. laevis* populations. Although it was argued, long ago, that some anurans in some environments transition through a hermaphroditic phase during development (Witschi 1929), this literature does not argue that *adult* amphibians commonly have oocytes within testicular tissue or are naturally hermaphroditic (Eggert 2004; Hayes 1998). Indeed, *X. laevis* sexually differentiates (without a transitional/hermaphroditic stage) during the larval period prior to sexual maturation (Iwasawa and Yamaguchi 1984). Thus, cases of gonadal abnormalities in “healthy” adult *X. laevis* populations should be rare. Given that simultaneous hermaphroditism has not been previously reported in *X. laevis* despite decades of research on their reproductive biology, an equally or more plausible explanation for high numbers of TOFs in control animals (e.g. Jooste et al. 2005; Orton et al. 2006) is exposure to some type of unmeasured endocrine disrupting contaminant.

Effects of Atrazine on Fish and Amphibian Sex Ratios

Given that atrazine exposure has been proposed to feminize gonadal development (Hayes et al. 2002), it might lead to female-biased sex ratios. Many studies, however, have severe methodological errors, such as contaminated controls, or inadequate data reporting (See Supplemental Material, Text, Table S1), preventing a conclusive synthesis of the effects of atrazine on sex ratios. None of the sex ratio studies used the most accepted and powerful approaches for testing for changes in sex ratios (e.g. Wilson and Hardy 2002). Only four studies, all on *X. laevis*, were of sufficient quality to be included in our meta-analysis and only one found that atrazine induced a female-biased sex ratio (See Supplemental Material, Table S2).

Effects of Atrazine on Fish and Amphibian Gonadal Function

Chemicals that alter gonadal development can affect gonadal function, such as germ cell (e.g. spermatogenesis in males) and steroid hormone production (McCoy et al. 2008; McCoy and Guillette in press), and thus can lead to altered reproductive success.

Effects on testicular cell types

Spermatogenesis is the process through which mature male gametes, spermatozoa, are produced from precursor cells (spermatogenic cells). The relative ratios of different spermatogenic cell types, rather than abundance of spermatozoa alone, is the most sensitive metric of altered spermatogenesis. Unfortunately, few studies on effects of atrazine on spermatogenesis met our inclusion criteria. Two of two studies demonstrated that atrazine was associated with altered spermatogenesis and that several cell types were affected (Table 6). Thus, atrazine appears capable of altering spermatogenesis, but the contexts and generality of these affects cannot be firmly established. Our analysis once again highlights a need for more rigorous investigations.

Effects on sex hormone concentrations

Sex hormone production is an important function of gonads that can be altered by gonadal abnormalities (McCoy et al. 2008). Indeed, altered hormone concentrations are the defining characteristic, in many cases, of "endocrine disruption". Six of seven studies on fish and amphibians document strong trends or significantly (five studies) altered sex hormone concentrations associated with atrazine exposure (Table 6). Although many of these studies were conducted in the field and are therefore correlative, the consistency of these results across studies suggests that atrazine alters sex hormone production and should be considered an endocrine disrupting chemical. A more thorough understanding of the effects of atrazine on hormone concentrations will require more detailed studies that account for the inherent variability endocrine system processes.

Effects on reproductive success

Reproductive success is strongly linked to population persistence and is likely one of the most important endpoints in toxicological studies. Five studies that evaluated the effects of atrazine on measures of reproductive success met our meta-analysis requirements (Table 6). Two studies on adult fish, *Pimephales promelas*, found no significant effect of atrazine on number of eggs produced, fertilization success, proportion of hatchlings, or larval development. However, one of these studies (Bringolf et al. 2004) found several non-significant, adverse trends (Table 6). Two of three studies on amphibians found no effects of atrazine on hatching success, whereas one showed reduced hatching success and delayed hatching (Table 6). Given the mixed results, the effect of atrazine on reproductive success needs to be studied in more thoroughly.

Effects of Atrazine on Fish and Amphibian Vitellogenin

Vitellogenin is an egg yolk precursor protein produced in the livers of female fish and amphibians. Estrogens induce vitellogenin synthesis in both males and females *in vivo* and quantification of vitellogenin is now an accepted screening test for estrogenic effects of chemicals (Scholz and Mayer 2008). None of the five studies (four on fish) found significant effects of atrazine on circulating or whole body concentrations of vitellogenin (See Supplemental Material, Table S2). Hence, these data do not support the hypothesis that atrazine is strongly estrogenic to fish.

Effects of Atrazine on Fish and Amphibian Aromatase

Cytochrome p450 aromatase catalyzes the conversion of androgens to estrogens in gonads and is critical for maintaining a balance between these sex hormone classes. Hayes et al. (2002) hypothesized that decreases in testosterone associated with atrazine exposure in their study could be driven by an atrazine-induced increase in aromatase and a concomitant increase in the conversion of testosterone and other androgens to estrogens. This hypothesis seemed reasonable because atrazine was known to increase aromatase in human cancer cell lines and in alligator gonadal-adrenal mesonephros (Crain et al. 1997; Sanderson et al. 2000). However, since 2002, several studies have explicitly tested whether atrazine increases aromatase in fish and amphibians, and only one of six studies included in our meta-analysis found that atrazine was associated with increased aromatase gene expression (See Supplemental Material, Table S2).

Effects of Atrazine on Fish and Amphibian Populations and Communities

Although there are too few studies examining the effects of atrazine on freshwater vertebrate populations to warrant meta-analysis and virtually all community-level studies infer, rather than test for, indirect effects (Rohr and Crumrine 2005), the effects of atrazine on populations and communities warrants a brief discussion. Any chemical that affects physiology, growth, development, reproduction, survival, or species interactions can affect population and community dynamics (Clements and Rohr 2009; Rohr et al. 2006a) However, the effects of contaminants might not result in immediate population declines because the survivors of chemical exposure frequently have less competition for resources, thus providing density-mediated compensation for adverse effects of the chemical (Rohr et al. 2006b). Demonstrating that a factor is the cause of any population decline is, indeed, incredibly difficult (Rohr et al. 2008a). Rohr et al. (2006b) revealed significant and delayed declines in *Ambystoma barbouri* salamander “populations” at 4, 40, and 400 µg/L of atrazine, above and beyond the counteracting effects of density-mediated compensation. Although this study provided greater ecological realism than many studies on atrazine, caution should be taken extrapolating these effects to populations in nature because this study was conducted in laboratory terraria. There is certainly a need for controlled studies on the effects of pesticides on wildlife populations.

Several studies have examined the effects of atrazine on amphibian and fish communities (Boone and James 2003; de Noyelles et al. 1989; Kettle 1982; Rohr and Crumrine 2005; Rohr et al. 2008c). Many of these studies reported alterations in fish or amphibian growth and abundance that seem to be caused by atrazine-induced changes in photosynthetic organisms (reviewed by Giddings et al. 2005; Solomon et al. 2008). At ecologically relevant concentrations, atrazine is expected to have a bevy of indirect effects by altering the abundance of periphyton, phytoplankton, and macrophytes (Huber 1993; Solomon et al. 1996). However, none of these studies distinguish between direct and indirect effects of atrazine on fish or amphibians.

There are several field studies comparing amphibian populations or species richness between atrazine-exposed and unexposed habitats (Bonin et al. 1997; Du Preez et al. 2005; Knutson et al. 2004). All of these studies are correlational and none thoroughly considered or ruled out alternative hypotheses for the observed patterns.

Caveats

We would be remiss to not mention some caveats regarding this meta-analysis. First, a problem with many meta-analyses is the "file-drawer" effect. This refers to the fact that researchers tend to place the results of experiments showing no effects in their file drawer and many journals tend to publish fewer studies showing no effects than effects (Gurevitch and Hedges 1993; Osenberg et al. 1999). This might be less of a problem in studies on pesticides because these chemicals are designed to kill biota and thus, in many cases, the null hypothesis might be an effect rather than the absence of one. Additionally, a substantial industry contingent works to ensure that both significant and non-significant effects of chemicals get published. Indeed, in the atrazine review by Solomon et al. (2008), there were approximately 63 cases where atrazine had significant adverse effects and 70 cases where atrazine had no significant effects (Rohr and McCoy in review), suggesting that the file-drawer effect is unlikely to be strongly biasing submission and publication of non-significant atrazine results. However, we cannot completely discount the possibility that the file-drawer effect generated a bias toward greater publication of significant effects of atrazine.

Another admonishment is that some of the endpoints in this meta-analysis were not independent of one another. For example, we tallied multiple endpoints from a single study despite the possibility that they might not be entirely independent.

Finally, we must consider the findings of this meta-analysis on atrazine relative to alternative strategies for weed control. If the alternative to atrazine is another chemical, then we should ideally compare the effects of atrazine to the replacement chemical. In fact, atrazine might be less detrimental to freshwater vertebrates than a replacement herbicide. If the alternative to atrazine does not entail a chemical replacement, then the effects revealed here might indeed be disconcerting. However, we also cannot ignore the benefit, if any, that atrazine provides. Interestingly, several studies estimate that atrazine only increases corn yields by 1-3% (reviewed by Ackerman 2007). To adequately evaluate any chemical, we should ideally conduct a thorough cost-benefit analysis that considers the focal chemical and alternatives to its use and that is based on comprehensive and accurate knowledge (see Ackerman 2007 for a review and critique of atrazine cost-benefit analyses).

Conclusions

Like past reviews, we found little evidence that atrazine consistently causes direct mortality of freshwater vertebrates at ecologically relevant concentrations, but there is evidence that atrazine might have adverse indirect ecological effects. However, in contrast to a previous review on atrazine (Solomon et al. 2008), we unveiled consistent effects of atrazine at ecologically relevant concentrations for many other response variables in our meta-analysis. The discrepancy between our findings and the conclusions of previous reviews could partly be a function of differences in criteria for including studies in the group used to draw general conclusions about atrazine effects.

Past reviews (e.g. Solomon et al. 2008) did not clearly define their inclusion criteria and did not make it clear which studies affected, or how they came to, their conclusions and regularly dismissed significant effects of atrazine.

Here, we revealed that, for freshwater vertebrates, atrazine consistently reduced growth rates, had variable effects on timing of metamorphosis that were often non-monotonic, elevated locomotor activity, and reduced anti-predator behaviors. Amphibian and fish immunity was reliably reduced by ecologically relevant concentrations of atrazine and this was regularly accompanied by elevated infections. Atrazine exposure induced diverse morphological gonadal abnormalities in fish and amphibians and was associated with altered gonadal function, such as modified sex hormone production. This suggests that atrazine should be considered an endocrine disrupting chemical. Finally, we do not have a thorough appreciation of the reproductive repercussions of atrazine.

Several endpoints had enough well-conducted studies to warrant more sophisticated meta-analyses based on effect sizes (e.g. growth, timing of metamorphosis, activity, immunity, infections, gonadal abnormalities). Meta-analyses based on effect sizes can provide parameter and standard errors estimates and thus can be useful for probabilistic risk assessment and for predicting atrazine effects.

Although we revealed consistent effects of atrazine on freshwater vertebrates, the consequences of these effects remain uncertain. We know little about how atrazine-induced changes in vertebrate growth, somatic development, behavior, immunity, gonadal development, or physiology affect reproduction, populations, gene frequencies, or communities. However, it was Sir Austin Bradford Hill who wisely stated in his address to the Royal Society of medicine in 1965 that:

> All scientific work is incomplete [and]…liable to be upset or modified by advancing knowledge. That does not confer upon us freedom to ignore the knowledge we already have, or to postpone action that it appears to demand at a given time (Hill 1965).

Whatever action is taken in the USEPA's re-evaluation of atrazine, we strongly encourage regulators to consider the consistent effects of atrazine on various taxa and to weigh these effects against any benefits atrazine provides and alternatives to atrazine use.

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Table 1. Summary of the results for the effects of atrazine on the developmental rate and size at or near metamorphosis for amphibians. Excluded studies can be found in Table S1.

		Net effect on developmental rate				Size at or near metamorphosis								
Taxon	Species	Effect direction	Conc. where effect was observed (μ/L)	Non-monotonic dose response[a]	Excluded from meta-analysis?	Effect direction	Conc. where effect was observed (μ/L)	Non-monotonic dose response[a]	Excluded from meta-analysis?	Conc. tested (μ/L)	Atrazine grade	Experiment type[b]	Exposure duration[c]	Reference
Frog	*Bufo americanus*	None detected	-	NA	No	Decreased	200	NA	No	200	Commercial; Aatrex[d]	PE	88 d or less	Boone and James 2003[e]
Frog	*B. americanus*	Decreased[f]	250, 500, 1000	Yes	No	Decreased[g]	No conc. differed from controls	No	No	250, 500, 1000, 5000, 10000	Technical	SR	3 wk	Freeman et al. 2005
Frog	*B. americanus*	None, trend toward decrease	-	No	No	Data not provided	-	Data not provided	Yes	1, 3, 30	Technical	SR	LTM	Storrs and Semlitsch 2008
Frog	*Rhinella arenarum*	Increased at 100 & 1000, decreased at 5000	100, 1000, 5000	Yes	No	Data not provided	-	Data not provided	Yes	100, 1000, 5000	Technical	SR	LTM	Brodeur et al. 2009
Frog	*Hyla chrysoscelis*	Increased	192	No	No	Data not provided	-	Data not provided	Yes	96, 192	Technical	PE, two pulses	129 d or less	Briston and Threlkeld 1998[e]
Frog	*H. versicolor*	None detected[h]	-	Possibly	No	Decreased	200, 2000	No	No	20, 200, 2000	Technical	PE	Mean of 13 d	Diana et al. 2000[i]
Frog	*H. versicolor*	None detected	-	NA	No	Data not provided	-	Data not provided	Yes	1, 3, 30	Technical	SR	LTM	Storrs and Semlitsch 2008
Frog	*Rana clamitans*	Decreased	10	Yes	No	Decreased	10	Yes	No	10, 25	Technical	SR	273 d or less	Coady et al. 2004[i]
Frog	*R. pipiens*	Unknown[j]	-	No	Yes	Decreased[k]	Not tested	No	No	20, 200	Technical	SR	LTM	Allran and Karasov 2000
Frog	*R. pipiens*	None detected	-	NA	No	Decreased	0.1	NA	No	0.1	Technical	SR	LTM	Hayes 2006

Frog	*R. pipiens*	None detected	-	NA	No	None, trend toward decrease under UV	-	NA	No	5	Not provided	SR	ETM, 45 d or less	Bridges et al. 2004[l]
Frog	*R. sphenocephala*	None detected	-	NA	No	Decreased	200	NA	No	200	Commercial; Aatrex[d]	PE	57 d or less	Boone and James 2003[e]
Frog	*R. sphenocephala*	None detected	-	NA	No	Data not provided	-	Data not provided	Yes	1, 3, 30	Technical	SR	LTM	Storrs and Semlitsch 2008
Frog	*R. sylvatica*	Data not provided	-	Data not provided	Yes	Decreased	Unknown, conc. in ponds not provided	NA	No	3, 30	Commercial	FS	Unknown	Kiesecker 2002[o]
Frog	*Xenopus laevis*	Data not provided	-	Data not provided	Yes	None, trend toward decrease	-	No	No	1, 10, 25	Technical	SR	Mean of 56 d	Carr et al. 2003
Frog	*X. laevis*	None detected	-	NA	No	Data not provided	-	Data not provided	Yes	1, 10, 25	Technical	SR	ETM	Du Preez et al. 2008
Frog	*X. laevis*	Increased	100, 450, 800	No	No	Unknown[p]	-	Unknown	Yes	100, 450, 800	Technical	SR	4 wk	Freeman and Rayburn 2005
Frog	*X. laevis*	Unknown[m,n,q]	-	Unkown	Yes	Decreased[r]	0.01, 1, 100	Possibly	No	0.01, 0.1, 1.0, 25, and 100	Technical	SR	75 d or less	Kloas et al. 2009
Frog	*X. laevis*	Decrease detected by regression	No conc. differed from controls	No	No	Decreased	20, 40, 80, 160, 320	No	No	20, 40, 80, 160, 320	Technical	SR	LTM	Sullivan and Spence 2003
Frog	*X. laevis*	Data not provided	-	NA	Yes	Decreased	400	NA	No	400	Technical	SR	LTM	Langerveld et al. 2009
Salamander	*Ambystoma barbouri*	Increased	40, 400	No	No	Decreased	400	No	No	4, 40, 400	Technical	SR	Mean of 52 d exposure	Rohr et al. 2004
Salamander	*A. macrodactylum*	Increased	184	No	No	Decreased	184	No	No	1.84, 18.4, 184	Technical	SR	30 d	Forson and Storfer 2006a

Salamander	*A. tigrinum*	Increased	16 vs 1.6, but not vs 0	Possibly, data not provided	No	None, trend toward decrease[s]	-	Data not provided	No	1.6, 16, 160	Technical	SR	LTM	Forson and Storfer 2006b
Salamander	*A. maculatum*	Increased and decreased[t]	250	Yes	No	Decreased	250	No	No	75, 250	Technical	SR	86 d	Larson et al. 1998
Salamander	*A. maculatum*	Decreased	200	NA	No	Decreased	200	NA	No	200	Commercial; Aatrex[d]	PE	57 d or less	Boone and James 2003[e]
Salamander	*A. texanum*	Decreased	200	NA	No	Decreased	200	NA	No	200	Commercial; Aatrex[d]	PE	88 d or less	Boone and James 2003[e,u]

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[a] NA = Not applicable, used when there were too few concentrations to evaluate non-monotonicity

[b] PE = Pulse experiment, SR = Static renewal experiment, FS= Field survevy

[c] LTM = Early larvae to metamorphosis, ETM = Embryo to metamorphosis, "or less" refers to cases where amphibians metamorphosed before atrazine exposure ceased

[d] Aatrex is 59.2% inactive ingredients

[e] Community-level study

[f] Authors show that atrazine modifies the thyroid axis for both *Xenopus laevis* and *Bufo americanus*

[g] All five atrazine concentrations tested reduced frog size relative to controls, but no within group variance estimates were provided

[h] 200 ppb developed faster than 2000 ppb

[i] Only a single egg mass, might not reflect general response

[j] Only use 50% of the metamorphs in the time to metamorphosis analysis without describing how they selected this subset of metamorphs or why they only used 50% for time to metamorphosis but 100% of the metamorphs for size at metamorphosis

[k] They report an interaction between atrazine and time for frog length, indicating that control animals were larger than those exposed to atrazine by the end of the experiment

[l] Tested as a mixture of 5 μ/L of atrazine and 5 μ/L of carbaryl

[m] Provide no within-group variance estimate

[n] No statistics provided but conclude that there was no effect of atrazine

[o] Compared ponds with and without atrazine, effects might be due to other factors

[p] Frogs lose weight at metamorphosis, and thus mass measurements were confounded by lumping tadpole and metamorph weights

[q] Graphs for developmental rate through time are indiscernible

[r] Only detected effects in one of two experiments and for females only

[s] *P*=0.080 for regression analysis, one-tailed test

[t] Results depended on developmental stage. Authors show that atrazine modifies thyroxine and corticosterone horomones

[u] Results depended on drying conditions

Table 2. Summary of the results for the effects of atrazine on fish and amphibian behaviors. Excluded studies can be found in Table S1.

Taxon	Species	Endpoint	Effect direction	Conc. where effect was observed (μ/L)	Conc. tested (μ/L)	Non-monotonic dose response[a]	Atrazine grade	Experiment type[b]	Exposure duration[c]	Reference
Locomotor activity										
Salamander	*Ambystoma barbouri*	Locomotor activity after disturbance	Increased	400	4, 40, 400	No	Technical	SR	37 d	Rohr et al. 2003
Salamander	*A. barbouri*	Locomotor activity after disturbance	Increased	400	4, 40, 400	No	Technical	SR	Mean of 52 d; LTM	Rohr et al. 2004
Salamander	*A. barbouri*	Locomotor activity after disturbance	Increased	40, 400	4, 40, 400	No	Technical	SR	Mean of 47 d; LTM	Rohr and Palmer 2005
Salamander	*A. barbouri*	Locomotor activity	Increased	400	40, 400, 800	No	Technical	PE	4 d	Rohr et al. unpublished data
Frog	*Rana sylvatica*	Locomotor activity	Increased	Two doses of 25 separated by two weeks	two doses of 25 separated by two weeks	NA	Technical	PE	1 mos	Rohr and Crumrine 2005[d]
Frog	*Bufo americanus*	Locomotor activity	None detected	-	201	NA	Technical	PE	4 d	Rohr et al. 2009
Frog	*Xenopus laevis*	Abnormal swimming	Increased	25	1, 10, 25	No	Technical	SR	Mean of 56 d, LTM	Carr et al. 2003
Frog	*Hyla chrysoscelis*	Burst swimming	Increased	Positive dose response	96, 192	No	Technical	PE, two pulses	129 d or less, LTM	Briston and Threlkeld 1998
Fish	*Carassius auratus*	Burst swimming	Increased	0.5, 50	0.5, 5, 50	Possibly	Technical	PE	1 d	Saglio and Tijasse 1998
Fish	*C. auratus*	Burst swimming	Increased	0.1, 1, 10	0.1, 1, 10	Possibly	Technical	PE	1 d	Saglio and Tijasse 1998

Fish	*Oncorhynchus mykiss*	Locomotor activity	Increased	1, 10	1, 10, 100	Yes	Technical	PE	30 min	Tierney et al. 2007
Fish	*Lepomis cyanellus*	Locomotor activity	Increased/ decreased	400 but not 800	40, 400, 800	Yes, only in presence of natural prey	Technical	PE	4 d	Rohr et al. unpublished data
Fish	larval *Sciaenops ocellatus*[f]	Locomotor activity and abnormal swimming	Increased	40, 80	40, 80	No	Technical	PE	72 h	Alvarez and Fuiman 2005
"Predation-related" risk reduction										
Sala-mander	*A.barbouri*	Refuge use	Decrease, detected with regression	None	4, 40, 400	No	Technical	SR	37 d	Rohr et al. 2003
Sala-mander	*A.barbouri*	Refuge use	Decreased	400	4, 40, 400	No	Technical	SR	Mean of 52 d, LTM	Rohr et al. 2004
Frog	*R. sylvatica*	Refuge use	Decreased	Two doses of 25 separated by two weeks	two doses of 25 separated by two weeks	NA	Technical	PE, two pulses	1 mos	Rohr and Crumrine 2005[d]
Fish	*C. auratus*	Grouping	Decreased	5, 50	0.5, 5, 50	No	Technical	PE	1 d	Saglio and Tijasse 1998
Fish	*C. auratus*	Sheltering in presence of predator cue	Decreased	5	0.5, 5, 50	Possibly	Technical	PE	1 d	Saglio and Tijasse 1998
Fish	*C. auratus*	Grouping in in presence of predator cue	Decreased	5	0.5, 5, 50	Possibly	Technical	PE	1 d	Saglio and Tijasse 1998
Fish	larval *S. ocellatus*[e]	Predation rates	None detected	40, 80	40, 80	No	Technical	PE	72 h	Alvarez and Fuiman 2005
Olfaction										

Frog	*B. americanus*	Chemical detection of food, parasites, & predator cues	None detected	-	201	NA	Technical	PE	4 d	Rohr et al. 2009
Sala-mander	*Plethodon shermani*	Chemical detection of food or sex pheromones	None detected	-	300	NA	Technical	SR	28 d	Lanzel 2008
Sala-mander	*P. shermani*	Activated olfactory neurons	None detected	-	700	NA	Technical	SR	28 d	Lanzel 2008
Fish	*Salmo salar*	Olfactory response (electroolfactogram)	Decreased	2, 5, 10, 20	0.1, 1, 2, 5, 10, 20	No	Technical	PE	30 min	Moore & Waring 1998
Fish	*S. salar*	Olfactory response (electroolfactogram)	Decreased	1	0.5, 1	No	Technical	PE	30 min	Moore & Lower 2001
Fish	*S. salar*	Olfactory response (electroolfactogram)	Decreased	0.5, 1	0.5, 1	No	Technical	PE	30 min	Moore & Lower 2001[f]
Fish	*O. mykiss*	Olfactory response (electroolfactogram)	Decreased	10, 100	1, 10, 100	No	Technical	PE	30 min	Tierney et al. 2007
Fish	*O. mykiss*	Response ratio to L-histidine	Decreased	10	1, 10, 100	Possibly	Technical	PE	30 min	Tierney et al. 2007
Other behaviors										
Sala-mander	*A. barbouri*	Water conserving behaviors	Decreased	40, 400	4, 40, 400	No	Technical	SR	Mean of 52 d; LTM	Rohr and Palmer 2005[g]

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[a] NA = Not applicable, used when there were too few concentrations to evaluate non-monotonicity

[b] PE = Pulse experiment, SR = Static renewal experiment

[c] LTM = Early larvae to metamorphosis

[d] Community-level study

[e] Larval red drum are often found in freshwater so they were included in this meta-analysis

[f] Mixture of 0.5:0.5 and 1.0:1.0 atrazine and simazine; thus total conc. of triazine was 1 and 2 ppb, respectively

[g] Increased salamander water loss and thus desiccation risk

Table 3. Summary of the results for the effects of atrazine, through water column exposure, on fish and amphibian immunity. Excluded studies can be found in Table S1.

Taxon	Species	Endpoint	Effect direction	Conc. where effect was observed (µ/L)	Conc. tested (µ/L)	Non-monotonic dose response[a]	Atrazine grade	Experiment type[b]	Exposure duration	Reference
Salamander	*Ambystoma tigrinum*	No. of peripheral leukocytes	Decreased	16, 160	1.6, 16, 160	No	Technical	SR	Until metamorphosis	Forson and Storfer 2006b
Frog	*Rana pipiens*	Splenocyte viability	None detected	-	2.1, 21, 210	No	Technical	SR	21 d	Christin et al. 2003, 2004[c]
Frog	*R. pipiens*	No. of splenocytes	Decreased, if use appropriate one-tailed test	210	2.1, 21, 210	No	Technical	SR	21 d	Christin et al. 2003, 2004[c]
Frog	*R. pipiens*	No. of phagocytic splenocytes	Decreased post-infection	210	2.1, 21, 210	No	Technical	SR	21 d	Christin et al. 2003[c]
Frog	*R. pipiens*	T-cell proliferation	Decreased in presence of mitogens	2.1, 21, 210	2.1, 21, 210	No	Technical	SR	21 d	Christin et al. 2003, 2004[c]
Frog	*R. pipiens*	T-cell proliferation	Decreased in absence of mitogens	2.1, 21, 210	2.1, 21, 210	No	Technical	SR	21 d	Christin et al. 2003, 2004[c]
Frog	*R. pipiens*	Absolute no. of phagocytic cells in spleen	Decreased	2.1, 21, 210	2.1, 21, 210	No	Technical	SR	21 d	Christin et al. 2004[c]
Frog	*R. pipiens*	No. of thymic plaques	Increased, indicating reduced immune capacity[d]	0.1	0.1	NA	Technical	SR	Until metamorphosis	Hayes et al. 2006

Frog	*R. pipiens*	No. of hemolytic plaques representing anti-body secreting B-cells	Decreased	1, 10	1, 10	No	Not provided	SR	4 wk	Houck and Sessions 2006
Frog	*R. pipiens*	No. of lymphocyte from spleen	None detected	-	1, 10	Possibly	Not provided	SR	8 wk	Houck and Sessions 2006
Frog	*R. pipiens*	No. of white blood cells	Decreased	0.01 to 10	0.01, 0.1, 1, 10	No	Technical	SR	8 d	Brodkin et al. 2007[e]
Frog	*R. pipiens*	No. of highly phagocytic cells	Decreased	0.01 to 10	0.01, 0.1, 1, 10	No	Technical	SR	8 d	Brodkin et al. 2007[e]
Frog	*Xenopus laevis*	Splenocyte viability	None detected, trend toward decrease at 7 d	-	2.1, 21, 210, 2100	No	Technical	SR	21 d	Christin et al. 2004[c]
Frog	*X. laevis*	Splenocyte cellularity	Decreased	210, 2100	2.1, 21, 210, 2100	No	Technical	SR	21 d	Christin et al. 2004[c]
Frog	*X. laevis*	Relative no. of phagocytic cells in spleen	Increased	21, 210, 2100	2.1, 21, 210, 2100	No	Technical	SR	21 d	Christin et al. 2004[c]
Frog	*X. laevis*	Absolute no. of phagocytic cells in spleen	Decreased	210, 2100	2.1, 21, 210, 2100	No	Technical	SR	21 d	Christin et al. 2004[c]
Frog	*X. laevis*	T-cell proliferation	None detected	-	2.1, 21, 210, 2100	Data not provided	Technical	SR	21 d	Christin et al. 2003[c]
Frog	*X. laevis*	Down-regulation of several genes involved in skin peptide defense	Decreased	400	400	NA	Technical	SR	Until meta-morphosis	Langerveld et al. 2009

Frog	*X. laevis*	Down-regulation of several genes involved in blood cell function	Decreased	400	400	NA	Technical	SR	Until meta-morphosis	Langerveld et al. 2009
Frog	*R. sylvatica*	No. of eosinophil from circulating blood	Decreased	3, 30	3, 30	No	Technical	SR	4 wk	Kiesecker 2002
Frog	*R. pipiens*	No. of melano-macrophages from liver	Decreased	<1, do not know max. conc.	Un-known	No	Commercial	FS	Unknown	Rohr et al. 2008c[f]
Frog	*R. paulustris*	No. of melano-macrophages from liver	Decreased	117	117	NA	Technical	PE	4 wk	Rohr et al. 2008c
Frog	*R. paulustris*	No. of eosinophil from liver	None detected, trend toward decrease, *p*=0.10	117	117	NA	Technical	PE	4 wk	Rohr et al. 2008c
Frog	*R. clamitans*	No. of eosinophil from liver	Decreased	117	117	NA	Technical	PE	4 wk	Rohr et al. 2008c
Frog	*R. clamitans*	No. of melano-macrophages from liver	None detected, trend toward decrease	117	117	NA	Technical	PE	4 wk	Rohr et al. 2008c
Fish	*Carassius auratus*	No. of superoxide radical from macrophages of spleen and kidney	Increased 4 and 8 weeks; "indicator of oxidative stress"	42	42	NA	Technical	SR	12 wk	Fatima et al. 2007[c]
Fish	*C. auratus*	Plasma lysozyme activity	Increased at 8 and 12 weeks, argued as a reduction in resistance to infection	42	42	NA	Technical	SR	12 wk	Fatima et al. 2007[c]
Fish	*C. auratus*	Antibody titres against *Aeromonas hydrophila*	Decreased	42	42	NA	Technical	SR	12 wk	Fatima et al. 2007[c]

Fish	*C. auratus*	Antioxidant enzyme in spleen (superoxide dismutase)	Decreased at 4, 8, and 12 weeks	42	42	NA	Technical	SR	12 wk	Fatima et al. 2007[c]
Fish	*Galaxias maculatus*	Leucocrit	Decreased	3, 50	0.9, 3, 10, 50	Possibly	Technical	SR	10 d	Davies et al. 1994
Fish	*Oncorhynchus mykiss*	Proliferative ability of circulating T lymphocytes (ConA)	Decreased	>5000	1000-10,000	Possibly	Technical	PE	2 d	Rymuszka et al. 2007
Fish	*O. mykiss*	Proliferative ability of circulating B lymphocytes (LPS)	Decreased	>5000	1000-10,000	Possibly	Technical	PE	2 d	Rymuszka et al. 2008
Fish	*O. mykiss*	Respiratory burst activity of circulating phagocytes	Decreased	>2,500	1000-10,000	Possibly	Technical	PE	2 d	Rymuszka et al. 2009
Fish	*Liza ramada* and *L. aurata*	Macrophage quality	Decreased (cells degenerated)	25-280	Un-known	Unknown	Unknown	Un-known	Unknown	Biagianti-Risbourg 1990[g]
Fish	*L. ramada* and *L. aurata*	Melanomacrophage centers in liver	Increased	25-280	Un-known	Unknown	Unknown	Un-known	Unknown	Biagianti-Risbourg 1990[g]
Fish	*Salmonidae* (species not specified)	White blood cells	Decreased	100-1000	Un-known	Unknown	Unknown	Un-known	Unknown	Walsh and Ribelin 1975[g]
Fish	*Salmonidae* (species not specified)	Lymphoid organ quality	Decreased (evidence of atrophy)	100-1000	Un-known	Unknown	Unknown	Un-known	Unknown	Walsh and Ribelin 1975[g]
Fish	*Salvelinus namaycush, O. kisutch*	Spleen weight	Decreased/ no effect	1500-13500	Un-known	Unknown	Unknown	Un-known	Unknown	Zeeman and Brindley 1981
Fish	*Salvelinus namaycush, O. kisutch*	Number of lymphocytes	Decreased/ no effect	1500-13500	Un-known	Unknown	Unknown	Un-known	Unknown	Zeeman and Brindley 1981

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[a] NA = Not applicable, used when there were too few concentrations to evaluate non-monotonicity

[b] PE = Pulse experiment, SR = Static renewal experiment, FS = Field survey

[c] Atrazine was a component of a mixture of pesticides tested and thus the experiment did not isolate the effects of atrazine
[d] Atrazine alone and every mixture containing atrazine increased thymic plaques
[e] Immune response stimulated by thioglycollate
[f] No quantified factors correlated with atrazine could parsimoniously explain patterns in infection
[g] As reported by Dunier and Swicki 1993; could not obtain original works

Table 4. Summary of the results for the effects of atrazine, through water column exposure, on fish and amphibian parasite infections. Excluded studies can be found in Table S1.

Taxon	Species	Endpoint	Effect direction	Conc. where effect was observed (μ/L)	Conc. tested (μ/L)	Non-monotonic dose response[a]	Atrazine grade	Experi-ment type[b]	Exposure duration	Reference
Sala-mander	*Ambystoma macrodactylum*	Infectivity of *Ambystoma tigrinum virus* (ATV)	Decreased	Not provided	1.84, 18.4, 184	Dose response not provided	Technical	SR	30 d	Forson and Storfer 2006a[c]
Sala-mander	*A. tigrinum*	Percent infected with ATV	Increase at 16 but not 1.6 or 160	16	1.6, 16, 160	Yes	Technical	SR	Until meta-morphosis	Forson and Storfer 2006b[d]
Sala-mander	*A. tigrinum*	Viral load	None detected, *p*=0.14	-	20, 200	No	Technical	SR	2 wk	Kerby and Storfer 2009
Sala-mander	*A. tigrinum*	Mortality due to ATV	Increased	Not provided	20, 200	No	Technical	SR	2 wk	Kerby and Storfer 2009
Frog	*Rana pipiens*	*Rhabdias ranae* nematode prevalence	None detected, trend toward increase	-	2.1, 21, 210	No	Technical	SR	21 d	Christin et al. 2003[e]
Frog	*R. pipiens*	No. of adult *Rhabdias ranae* nematode	Increased, clear dose response	21+210 > controls, 210 > water control	2.1, 21, 210	No	Technical	SR	21 d	Gendron et al. 2003[e]
Frog	*R. pipiens*	*Chryseobacterium* (Flavobacterium) *menigosepticum* infections	Increased	0.1	0.1	NA	Technical	SR	Until meta-morphosis	Hayes et al. 2006[e.f]
Frog	*R. pipiens*	*Rhabdias ranae* nematode within host migration	Faster	21, 210	2.1, 21, 210	No	Technical	SR	21 d	Gendron et al. 2003[e]

Frog	*R. pipiens*	*Rhabdias ranae* nematode maturation and reproduction	Earlier	21, 210	2.1, 21, 210	No	Technical	SR	21 d	Gendron et al. 2003[e]
Frog	*R. sylvatica*	No. of *Ribieoria* sp. and *Telorchis* sp.	Increased	3, 30	3, 30	No	Technical	SR	4 wk	Kiesecker 2002
Frog	*R. sylvatica*	Limb deformities caused by *Ribieoria* sp.	Increased in ponds with atrazine	ponds with atrazine	un-known	NA	Commercial	FS	Unknown	Kiesecker 2002
Frog	*R. clamitans*	No. of *Echinostoma trivolvis* cercariae	Increased	201	201	NA	Technical	SR	2 wk	Rohr et al. 2008b[g]
Frog	*R. pipiens*	No. of larval trematodes	Increased	<1, but do not know max. conc.	un-known	No	Commercial	FS	Unknown	Rohr et al. 2008c[h]
Frog	*R. clamitans*	No. of larval *Plagiorchid* trematodes	Increased	117	117	NA	Technical	PE	4 wk	Rohr et al. 2008c
Frog	*R. clamitans*	No. of *Echinostoma trivolvis* cercariae	Decreased, but amphibians not exposed to atrazine	20, 200	20, 200	No	Commercial; Aatrex[i]	PE	Cercariae exposed for 2h	Koprivnikar et al. 2006[j,k,l]
Fish	*Carassius auratus*	Mortality due to *Aeromonas hydrophila* challenge	Increased	42	42	NA	Technical	SR	12 wk	Fatima et al. 2007[e]

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[a] NA = Not applicable, used when there were too few concentrations to evaluate non-monotonicity

[b] PE = Pulse experiment, SR = Static renewal experiment, FS = Field survey

[c] Effect was observed when combining of 1.84, 18.4, and 184 treatments and compariong to controls, effect might be predominantly due to 184

[d] 160 ppb was thought to reduce ATV infectivity explaining non-monotonicity

[e] Atrazine was a component of a mixture of pesticides tested and thus the experiment did not isolate the effects of atrazine

[f] Only saw this effect when atrazine was mixed with eight other pesticides

[g] Effect was found pooling pesticides and comparing them to control treatments
[h] No quantified factors correlated with atrazine could parsimoniously explain patterns in infection
[i] Aatrex is 59.2% inactive ingredients
[j] Effects could be due to inactive ingredients
[k] Effects could be due to chemicals other than atrazine that might be in the pond water used to make the stock solutions
[l] All LC50s were calculated incorrectly

Table 5. Summary of the effects of atrazine on general gonadal morphology. Excluded studies can be found in Table S1.

Taxon	Species	Endpoint	Effect direction	Conc. where effect was observed (μ/L)	Conc. tested (μ/L)	Atrazine grade	Experi-ment type[a]	Exposure duration	Reference
Testes									
Fish	*Pimephales promelas*	Testis size corrected for body size	Trend for decrease	5, 50	5, 50	Technical	SR	21 days	Bringolf et al. 2004[b]
Frog	*Xenopus laevis*	Discontinuous gonads (abnormal segmentation)	Increased	25	1.0, 10, 25	Technical	SR	~78 days during larval period	Carr et al 2003
Frog	*X. laevis*	Ambiguous gonads (not obviously male or female)	Increased	25	1.0, 10, 25	Technical	SR	~78 days during larval period	Carr et al 2003[c]
Frog	*X. laevis*	Testis size corrected for body size	Increased	10	10, 100	Technical	SR	48 days	Hecker et al. 2005a[b]
Frog	*X. laevis*	Sperm/area	None	-	10, 100	Technical	SR	48 days	Hecker et al. 2005a[b]
Frog	*X. laevis*	Testis size corrected for body size	None	-	1, 25, 250	Technical	SR	36 days	Hecker et al. 2005a[b]
Frog	*Rana clamitans*	Testis size corrected for body size	Decreased in juvenile males	ND[d]-3.13	ND[d]-3.13 (see comments)	Commercial	FS	Unknown	McDaniel et al. 2008[e]
Frog	*R. pipiens*	Testicular ovarian follicles (testicular oocytes)	Increased where atrazine was detected in 2003 (but see [e])	ND-3.14	ND-3.13 (see comments)	Commercial	FS	Unknown	McDaniel et al. 2008[e,f]
Frog	*various spp., mostly R. clamitans*	Discontinuous testes (abnormal segmentation)	None	-	ND-2[g]	Commercial	FS	Unknown	Murphy et al. 2006a
Frog	*various spp., mostly R. clamitans*	Intersex (having testicular and ovarian tissues)	None	-	ND-2[g]	Commercial	FS	Unknown	Murphy et al. 2006a

Frog	*various spp., mostly R. clamitans*	Testicular ovarian follicles (testicular oocytes)	Increased in one of two years in juveniles, positively correlated with max. atrazine conc. in that year	ND-0.73	ND-2[g]	Commercial	FS	Unknown	Murphy et al. 2006a
Frog	*R. clamitans*	Testis size corrected for body size	Increased in adult males at agricultural sites in one of two years	ND-250	ND-2[g]	Commercial	FS	Unknown	Murphy et al. 2006b[h]
Frog	*X. laevis*	Hermaphroditism (testicular oocytes, intersex, mixed sex)	None	-	0.1, 1, 10, 100	Technical	SR	~65 days during larval period	Oka et al. 2008
Frog	*Acris crepitans*	Intersex or testicular oocytes	Trend for increase	atrazine detections	ND-70	Commercial	FS	Unknown	Reeder et al. 1998[i]
Fish	*P. promelas*	Spermatogenic tubule diameter	Reduced	250	25, 250	Technical	FT	21 days	USEPA 2005
Ovaries									
Fish	*P. promelas*	Ovary size corrected for body size	Trend for decrease	50	5, 50	Technical	SR	21 days	Bringolf et al. 2004[b]
Frog	*Hyla versicolor, R. sphenocephala*	Ovarian developmental stage	None	-	1, 3, 30[j]	Technical	SR	Through metamorphosis	Storrs and Semlitsch 2004
Frog	*Bufo americanus*	Ovarian developmental rate	None	-	1, 3, 30[j]	Technical	SR	Through metamorphosis	Storrs and Semlitsch 2004
Fish	*P. promelas*	Proportion of oocytes undergoing atresia	None	-	25, 250	Technical	FT	21 days	USEPA 2006

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[a] FS = Field study, FT = Flow through experiment, PE = Pulse experiment, SR = Static renewal experiment

[b] No test statistics or degrees of freedom are presented. However, means and variances are presented in the text or in a figure.

[c] Xenopus are typically sexually differentiated at the gross morphological level at metamorphosis. Individuals in this study exposed to 25 µ/L were so sexually ambiguous they were initially considered intersexes (having both testicular and ovarian issues).

[d] ND = Nondetectable

[e] Atrazine concentration for the non-agricultural reference site during 2003 is reported incorrectly. Repeated attempts to contact the author for clarification have not been forthcoming.

[f] When atrazine concentrations were highest (2003), testicular ovarian follicles (TOF) per individual occurred in higher numbers. TOFs were positively associated with atrazine, nitrate, and quantity of pesticides in a multivariate comparison suggesting that atrazine is contributing to TOFs.

[g] Concentrations were between ND and 2 except on two occasion at one site when levels were 65 and 250 µ/L.

[h] Authors argue that differences in gonadal somatic index (GSI) between agricultural and non-agricultural sites cannot be due to atrazine because GSI does not correlate with atrazine concentration. However, no statistics are presented to support this claim.

[i] The relationship between detection of atrazine and the presence of one or more intersex cricket frogs approached significance (p = 0.07).

[j] Actual concnetrations of the 30 ug/L treatment was 125ug/L.

Table 6. Summary of the effects of atrazine on gonadal function. Excluded studies can be found in Table S1.

Taxon	Species	Endpoint	Effect direction	Conc. where effect was observed (µ/L)	Conc. tested (µ/L)	Atrazine grade	Experi-ment type[a]	Exposure duration	Reference
Testicular cell types									
Frog	*Rana clamitans*	Proportion of juvenile males with > 50% tubules containing spermatids and spermatozoa	Lower at agricultural site with highest atrazine concentrations	median range.068 -0.78	ND-3.13[b]	Commercial	FS	Unknown	McDaniel et al. 2008[b]
Frog	*R. pipiens*	Proportion of juvenile males with > 50% tubules containing spermatids and spermatozoa	Higher at agricultural site with highest atrazine concentrations	0.342 (mean of medians conc.)	ND-3.13[b]	Commercial	FS	Unknown	McDaniel et al. 2008[b]
Fish	*Pimephales promelas*	Proportion of primary spermatogonia	Increased	25, 250	25, 250	Test	FT	21 d	USEPA 2005
Fish	*P. promelas*	Proportion of secondary spermatogonia	Reduced	25, 250	25, 250	Test	FT	21 d	USEPA 2005
Sex hormone concentrations									
Frog	*Xenopus laevis*	Testosterone in adult males	Decreased	25	25	Technical	SR	46 d	Hayes et al. 2002[c]
Frog	*X. laevis*	Testosterone in adult males	None	-	10, 100	Technical	SR	48 d	Hecker et al. 2005a
Frog	*X. laevis*	Estradiol in adult males	None	-	10, 100	Technical	SR	48 d	Hecker et al. 2005a
Frog	*X. laevis*	Estradiol in adult males	None	-	1, 25, 250	Technical	SR	36 d	Hecker et al. 2005b
Frog	*X. laevis*	Testosterone in adult males	Decreased	250	1, 25, 250	Technical	SR	36 d	Hecker et al. 2005b

Frog	*X. laevis*	Testosterone in females	Decreased at agricultural sites, negatively correlated with conc. of atrazine & breakdown product	<0.1-4.14	<0.1-4.14	Commercial	FS	Unknown	Hecker et al. 2004
Frog	*X. laevis*	Testosterone in males	Negatively correlated with diaminochlorotriazine concentration (a product of atrazine breakdown)	<0.1-4.14	<0.1-4.14	Commercial	FS	Unknown	Hecker et al. 2004
Frog	*X. laevis*	Estradiol in females	Decreased at agricultural sites, negatively correlated with conc. of atrazine & breakdown product	<0.1-4.14	<0.1-4.14	Commercial	FS	Unknown	Hecker et al. 2004
Frog	*R. pipiens*	Testosterone in juvenile males (2003)	Decreased at agricultural sites	median range 0.380-0.780	ND-3.13[b]	Commercial	FS	Unknown	McDaniel et al. 2008[b]
Frog	*R. pipiens*	Testosterone in juvenile males (2003)	Negatively correlated with atrazine concentration	ND-3.13	ND-3.13[b]	Commercial	FS	Unknown	McDaniel et al. 2008[b,d]
Frog	*R. pipiens*	11-ketotestosterone in juvenile males (2003)	Negatively correlated with atrazine concentration	ND-3.13	ND-3.13[b]	Commercial	FS	Unknown	McDaniel et al. 2008[b,d]
Frog	*R. pipiens*	Testosterone in adult females (2003)	Negatively correlated with atrazine concentration	ND-3.13	ND-3.13[b]	Commercial	FS	Unknown	McDaniel et al. 2008[b,d]

Frog	*R. clamitans*	11-ketotestosterone to testosterone ratio in adult females (Late summer Aug-Sept. 2002)	Increased at agricultural sites	ag. sites ranged from ND-250	ND-250	Commercial	FS	Unknown	Murphy et al. 2006b[e]
Frog	*R. clamitans*	11-ketotestosterone to testosterone ratio in adult males (Late summer Aug.-Sept. 2002)	Increased at agricultural sites	ag. sites ranged from ND-250	ND-250	Commercial	FS	Unknown	Murphy et al. 2006b[e]
Frog	*R. clamitans*	11-ketotestosterone to testosterone ratio in adult males (Early summer May 2003)	Increased at agricultural sites	ag. sites ranged from ND-0.73	ND-250	Commercial	FS	Unknown	Murphy et al. 2006b[e]
Frog	*R. clamitans*	Estradiol to testosterone ratio in adult females (Late summer Aug.-Sept. 2002)	Increased at agricultural sites	ag. sites ranged from ND-250	ND-250	Commercial	FS	Unknown	Murphy et al. 2006b[e]
Frog	*R. clamitans*	Estradiol to testosterone ratio in adult males (Late summer Aug.-Sept. 2002)	Increased at agricultural sites	ag. sites ranged from ND-250	ND-250	Commercial	FS	Unknown	Murphy et al. 2006b[e]
Frog	*R. clamitans*	Estradiol to testosterone ratio in adult males (Early summer May 2003)	Decreased at agricultural sites	ag. sites ranged from ND-0.73	ND-250	Commercial	FS	Unknown	Murphy et al. 2006b[e]
Frog	*R. clamitans*	Estradiol to testosterone ratio in juvenile males (July 2003)	Increased at agricultural sites	ag. sites ranged from ND-0.73	ND-250	Commercial	FS	Unknown	Murphy et al. 2006b[e]
Frog	*R. clamitans*	Testosterone in adult males (Early summer May 2003)	Increased at agricultural sites	ag. sites ranged from ND-0.73	ND-250	Commercial	FS	Unknown	Murphy et al. 2006b[e]
Frog	*R. clamitans*	Testosterone in juvenile females (July 2003)	Increased at agricultural sites	ag. sites ranged from ND-0.73	ND-250	Commercial	FS	Unknown	Murphy et al. 2006b[e]

Frog	*R. clamitans*	Testosterone in juvenile males (July 2003)	Increased at agricultural sites (see comment)	ag. sites ranged from ND-0.73	ND-250	Commercial	FS	Unknown	Murphy et al. 2006b[e]
Fish	*P. promelas*	Testosterone female	None	-	25, 250	Technical	FT	21 d	USEPA 2005
Fish	*P. promelas*	Estradiol female	Trend (up to a 44% decrease)	25, 250	25, 250	Technical	FT	21 d	USEPA 2005[f]
Fish	*P. promelas*	Testosterone male	Trend (up to a 31% decrease)	25, 250	25, 250	Technical	FT	21 d	USEPA 2005[f]
Fish	*P. promelas*	11-ketotestosterone male	Trend (up to a 47% decrease)	25, 250	25, 250	Technical	FT	21 d	USEPA 2005[f]
Reproductive success									
Sala-mander	*Ambystoma barbouri*	Proportion hatched and timing of hatching	None	-	4, 40, 400	Technical	SR	37 d	Rohr et al. 2003
Sala-mander	*A. barbouri*	Proportion hatched and timing of hatching	Decreased and delayed hatching	400	4, 40, 400	Technical	SR	Mean of 52 d	Rohr et al. 2004
Frog	*R. pipiens*	Proportion hatched	None	-	2590-20,000	Technical	SR	10 d	Allran and Karasov 2001
Frog	*R. clamitans*	Proportion hatched	None	-	2590-20,001	Technical	SR	10 d	Allran and Karasov 2001
Frog	*Bufo americanus*	Proportion hatched	None	-	2590-20,002	Technical	SR	10 d	Allran and Karasov 2001
Fish	*P. promelas*	Eggs per spawning of exposed adults	Trend for a decrease	5	5, 50	Technical	SR	21 d	Bringolf et al. 2004[c]
Fish	*P. promelas*	Number of spawnings of exposed adults	Trend for a decrease	50	5, 50	Technical	SR	21 d	Bringolf et al. 2004[c]
Fish	*P. promelas*	Fertilization success of exposed adults	Trend for a decrease	50	5, 50	Technical	SR	21 d	Bringolf et al. 2004[c]

Fish	*P. promelas*	Proportion hatched and larval development of offspring from exposed adults	None	-	5, 50	Technical	SR	21 d	Bringolf et al. 2004[c]
Fish	*P. promelas*	Egg production of exposed adults	None	-	25, 250	Technical	FT	21 d	USEPA 2005
Fish	*P. promelas*	Fertilization success of exposed adults	None	-	25, 250	Technical	FT	21 d	USEPA 2005
Fish	*P. promelas*	Proportion hatched and larval development of offspring from exposed adults	None	-	25, 250	Technical	FT	21 d	USEPA 2005

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[a] FS = Field study, FT = Flow through experiment, SR = Static renewal experiment

[b] Atrazine concentration for the non-agricultural reference site during 2003 is reported incorrectly. Repeated attempts to contact the author for clarification have not been forthcoming.

[c] No test statistics or degrees of freedom are presented. However, means and variances are presented in the text or in a figure.

[d] Authors report no significant correlation between atrazine and sex hormones in their abstract when, in fact, these endpoints are negatively correlated. The negative correlations across sexes and age groups reported in this study are unlikely to occur due to a low sample size or sampling error as argued by the authors.

[e] Authors argue that differences in hormone levels between agricultural and non-agricultural sites cannot be due to atrazine because hormone concentrations do not correlate with atrazine concentration. However, no statistics are presented to support this claim.

[f] Low samples sizes (7-8 fish) likely precluded detecting these considerable effects.