Syngenta's Comments on the "Save the Frogs" EPA Petition Docket EPA-HQ-OPP-2011-0586

November 14, 2011

1 Executive Summary

Syngenta Crop Protection, LLC provides this response to the petition of docket EPA-HQ-OPP-2011-0586 titled "SAVE THE FROGS! Submission to the US Environmental Protection Agency". In summary;

- Atrazine and its potential impacts on amphibian sexual development underwent a detailed evaluation in 2003 by the EPA (USEPA 2003b). This EPA review included several studies cited in the petition summary. The EPA concluded in 2007 that "reproductive fitness (sex ratio, intersex condition) were unaffected" (USEPA 2007a) and similar conclusions were reached by other regulatory bodies including Australia Pesticides and Veterinary Medicines Authority (APVMA 2008).

- None of the information or references cited in the recent petition justifies a change in the regulatory status of atrazine. There were only a few studies referenced on amphibians and reptiles which were completed after EPA's 2007 review, and none of these provide compelling scientific evidence that would support this petition.

- Subsequent to the 2007 review by EPA, there have been several relevant publications from independent laboratories which were omitted from the petition which indicate no effects of atrazine on amphibians.

- The majority of the mammalian studies cited in the petition were designed to define mode-of-action, metabolism, and toxicological endpoints. They were not designed to, nor should they be used to, predict risk to aquatic species. The studies consistently demonstrate that effects in mammalian test systems only occur at high-dose levels that are not relevant to environmental concentrations

- All of the epidemiological studies in the petition have been reviewed by EPA and other regulatory bodies around the world and do not provide evidence of any potential for effects on reproductive health or cancer.

In conclusion, USEPA should reject the petition of docket EPA-HQ-OPP-2011-0586.

2 Introduction

Syngenta Crop Protection, LLC appreciates the opportunity to provide The United States Environmental Protection Agency with our response to the petition of docket EPA-HQ-OPP-2011-0586 titled “Save The Frogs! Submission to the US Environmental Protection Agency” (herein referred to as “the petition”).

Atrazine has been used as a herbicide since the end of the1950s and is important for the production of a number of crops, including corn, sorghum, and sugarcane. Since its registration, the potential effects and risks of atrazine to humans and wildlife have been reviewed by a number of regulatory agencies, including the U.S. EPA.

The potential effects of atrazine in wildlife (including amphibians) have been the subject of detailed and thorough reviews by the U.S. EPA (USEPA 2003a, 2003b, 2007a, 2007b). The conclusion with regard to amphibians was that “…reproductive fitness (sex ratio, intersex condition) was unaffected…” by atrazine (USEPA 2007b).

Other agencies have also conducted thorough and detailed review of the potential effects of atrazine on wildlife and have come to similar conclusions. The Australian Pesticides & Veterinary Medicines Authority (APVMA) stated that “…current data indicate that it is unlikely that atrazine is impacting adversely on Australian amphibian populations at current levels of exposure…” and it “…is satisfied that the continued use of products containing atrazine meets the criteria for continued registration and label approval as prescribed by the Agvet Codes…” (APVMA 2008). Similarly the Pest Management Regulatory Agency in Canada (PMRA) concluded that “Based on the review of available information, the PMRA has concluded that the use of atrazine and associated end-use products does not pose an unacceptable risk to the environment.” (PMRA 2007). In a review of one of the main studies (Hayes et al. 2002) cited in the petition, the UK regulatory agency concluded that “the paper grossly overstates the potential effects on frogs and changes in populations based on the data given” (UK 2003) .

The following response is focused on scientific information and the application of scientific principles of causality to the information referenced in the petition. This docket submission includes information from papers relevant to amphibian, fish, reptiles and mammals including:

- Critique of citations from the petition for amphibians, fish and reptiles.
- Additional details on certain publications cited in the petition published since the last amphibian Scientific Advisory Panel (SAP) of 2007
- Discussion of additional studies not included in the petition published since the last amphibian SAP of 2007.
- Information from mammalian toxicology studies and mode of action studies that were cited in the petition.

3 Critique of citations from the petition for amphibians, fish and reptiles.

Table 1 addresses areas of study cited in the petition for amphibians, fish and reptiles. In summary;

- Many of the studies cited in support of the petition have flaws in experimental design and interpretation, including:
    - The use of unrealistic experimental exposures relative to environmental exposures.
        - Extensive sampling, analysis, and modeling have shown that almost all (99%) are less than 20 µg/L in ponds and other lentic waters (Giddings et al. 2005).
    - The use of insufficient exposure concentrations or doses to allow proper interpretation of dose-response.
    - Lack of use of validated methods and study designs in highly variable test systems (frogs, salamanders, reptiles, etc), leading to study results that are not able to be replicated.
    - Lack of clear problem formulations and testable hypotheses.
    - Use of inappropriate assumptions/measurements with respect to timing of exposures.

- Many of the studies omit key information on methodology, data descriptions and/or did not provide sufficient exposure data to support the claims, including:
    - Lack of analytical confirmation of exposures during the study.
    - Insufficient or no information on husbandry and condition of the animals.
    - Incomplete descriptions of the methods used.
    - Lack of the adherence to good scientific practices, including Good Laboratory Practice (GLP) and Quality Assurance.
    - Incomplete “supplemental information”.

- Many of the studies provided by the petitioners contain incorrect data analyses and erroneous data interpretation, including:
    - Incorrect statistical analyses and comparisons.
    - Inappropriate transformation of data in concentration response studies of survival (e.g. corrections for mortality in the control not used).
    - Absence of consistent dose-responses.
    - Incorrect calculations.
    - Interpretation of the data beyond the study observations.

4 Additional details on certain publications cited in the petition published since the last amphibian Scientific Advisory Panel (SAP) of 2007

Of the amphibian and reptile studies listed in the petition, most have already been reviewed or available for review by EPA and/or the EPA Scientific Advisory Panel in 2003 and 2007. Subsequent to the formal EPA review in 2007, several additional papers concerning atrazine's potential effects on amphibians have been published, some of which are identified in the petition. These include: Hayes et al. (2010), Langlois et al. (2009), Rohr and McCoy. (2010b) Rohr et al. (2008b), Oka et al. (2008). A new publication by Tillitt et al.(2010) is also cited (incorrectly) by Hayes relative to effects on fish.

Hayes cites Oka et al. 2008 in the petition summary, stating that this paper supports his claim that "atrazine also completely feminizes" amphibians. This is not supported by the statistical analysis conducted by Oka et al. or their stated conclusions in the paper. These authors conclude that "higher female ratios in atrazine exposure groups in the present study were not caused by the estrogenic action of atrazine, since there is no evidence on induction of P450 aromatase gene in gonad, hepatic VTG induction, and the existence of hermaphroditic gonad."

The Hayes et al. (2010) purports to demonstrate that atrazine alters the sexual development of amphibians is weakened by design limitations, inconsistency with previous Hayes publications and based on a refuted mode of action.

- Previous Hayes publications were included in reviews on two separate occasions by the USEPA and Scientific Advisory Panels (2003 and 2007) and the studies' hypotheses were not supported. Given that these refuted effects of atrazine on sexual development are the biological basis and foundation of the current publication, the effects reported in Hayes et al. (2010) are questionable.
- This publication reports on a study that suffers from serious design limitations, such as including only a single dose level and the lack of a positive control necessary for system validation, comparison of effects, and demonstration of biological plausibility.
- Sampling and statistical methods are not clearly reported in many cases, making evaluation of the study difficult.
- Biological findings are not consistent with previous publications by Hayes.
- Many of the studies cited in this publication to support their work have not withstood rigorous scientific review by independent science panels and regulatory agencies and biological plausibility of key reported effects in this study is not demonstrated.

Langlois et al. (2009) suffers from design and conduct limitations and the results do not support the conclusions of the paper.

- The reported effects on sex ratio are likely due to variation in starting sex ratios which deviated widely from the nominal 50:50 male to female ratio and the result of small sample sizes. A relatively small subset of the total available frogs was selected for sex identification. Frogs failing to undergo metamorphosis were also excluded. The reported effects on sex ratio by atrazine exposure are inconsistent with numerous recent publications.
- Reported effects of atrazine and the positive control (EE2) on mortality and metamorphosis are inconsistent with previous work, including in the case of EE2, with studies conducted in the same laboratory.
- The methods used in this study were not validated by EE2, which showed no effects at concentrations where it has been widely demonstrated in numerous studies. The authors' statement that atrazine causes feminization at a concentration of (1.8 µg/L) is therefore not supported. The lack of estrogenic activity by atrazine has been well documented.
- A number of study design and study conduct limitations and uncertainties impair the strength and utility of this study.
    - The positive EE2 control failed to confirm the sensitivity of the system to feminization via estrogenic compounds, resulting in questionable findings relative to sex ratios.
    - The use of formulated atrazine, rather than atrazine active ingredient, confound the interpretation of the results.
    - The study was conducted with only two concentrations, not enough to properly test the hypotheses stated by the authors.
    - Important physiochemical parameters (eg. pH and dissolved oxygen) were not reported during the first two weeks of the study and therefore cannot be assessed relative to potential impacts on frogs in this period of the study. When monitored, varying numbers of tank replicates were sampled (between 2 to 5) and treatment means reported, thus tank variability is not provided.

- Given these limitations, the results of this study do not support the conclusion that atrazine alters gonadal differentiation and metamorphosis in developing *R. pipiens.*

Rohr and McCoy (2010b) suffers from multiple flaws and limitations and therefore the conclusions are not supported.

- This publication reports on a "qualitative meta-analysis" of existing data. Procedurally, the authors state "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 simply tallies the number of studies in which the authors determined did or did not exhibit an effect of atrazine.
- On the basis of the conclusions of Rohr and McCoy, atrazine is purported to be present at unrealistically high concentrations in the surface waters, and to adversely affect over 12 varying biological endpoints. To have such divergent mechanisms of

action all in a single substance and at low exposures would be unprecedented and is without explanation.

- A qualitative meta-analysis can be used to formulate hypotheses and theories but should not be used to make conclusions. The qualitative aspect of the meta-analysis performed by Rohr and McCoy is demonstrated by the fact that there is not an *a priori* hypothesis of what effects they are considering in their meta-analysis.
- There are several aspects of the paper that are judgmental rather than exploratory or hypothesis generating. For example, the title states "…reveals consistent effects…" which is a conclusion. In fact when they "paraphrase" USEPA's "definition" of meta-analysis as "...a systematic analysis of studies examining similar endpoints to draw general conclusions, develop support for hypotheses, and/or produce an estimate of overall effects", this is really a description of <u>quantitative</u> meta-analysis and not <u>qualitative</u> meta-analysis.
- In their qualitative meta-analysis, the authors did not take account of the effects of differences in species, differences in magnitude of the effects, differences in exposures, differences in experimental environments, differences in endpoints, etc.

In Rohr et al. (2008), key data were not measured or provided and their conclusions are inconsistent with expectations from numerous other studies.

- Key data from the field and mesocosm study were not measured nor provided, thereby questioning the reliability of the authors' causal inferences and overall conclusions that agrochemical exposure is linked to increased trematode infections in amphibians.
- The authors did not provide sufficient supporting data from the field or mesocosm studies within the main publication or accompanying supplementary information. Independent analysis of these data by interested stakeholders (including the public and regulatory agencies) are critical for evaluating dataset robustness and determining the significance, reproducibility, and reliability of the authors' statistical correlations, path analyses, and/or significance tests.
- Based on an extensive set of laboratory and mesocosm studies for atrazine, direct effects of atrazine on freshwater algal species will not occur at atrazine concentrations (≤0.59 µg/L) measured within the 18 Minnesota wetland sites.
- Contrary to the authors' assertion, desethylatrazine is orders of magnitude less toxic than parent atrazine to freshwater algae.
- Published findings from previous studies by Rohr *et al.*, as well as other researchers, are inconsistent with this study.
- Available data from a comprehensive set of mammalian studies do not support the conclusion that low concentrations of atrazine or desethylatrazine directly affects the immune system of vertebrates, including amphibians.

In Tillitt et al. (2010), study design limitations exist and the reported findings are inconsistent with other known studies.

- The results reported by Tillitt et al. (2010) are inconsistent with the results of 4 fish full life cycle studies which report no effects on reproduction, two of which were for the same species (fathead minnow) of fish.
- These 4 studies were conducted using a standard protocol and accepted by the USEPA. They are of much longer duration (274-450 days) and much higher atrazine exposures (maximum exposures 95 to 2000 ppb) and fish are continuously exposed to the test product from an early life stage (embryo/larval/juvenile) throughout development and reproductive stages until a pre-determined number of spawns occur. Endpoints include:
    - F_0: embryo hatching success, survival, length, weight, # eggs/spawn, total # of eggs, # spawns/female, # eggs/female
    - F_1: hatching success, survival, length, weight
- Although the study was well conducted, the weaknesses in the design relate to the deviations from the standard protocol in terms of the number of fish per replicate, the subsampling of half the fish at 14 d, and the retention of data from replicates in which one of the fish may have been reproductively compromised at the start of the study.
- These differences in the design do not allow the results of this study to be directly compared to two previous studies which followed the standard protocol and did not report statistically or biologically relevant responses at greater range of exposure-concentrations.
- The effect on production of eggs between 14 and 30 days could have been an artifact of the design and the inclusion of some fish with impaired development of the gonads.
- The publication overstates the results and the mechanism proposed for the alleged effect is not supported by the data. For example, the publication suggests that atrazine may have affected final oocyte maturation leading to a decrease in the number of eggs yet the data fail to show an effect on the proportion of fish with stage V follicles.
- Considering these points, the weight of evidence suggests that the overall conclusion is that there is either no or only a weak effect of atrazine on production of eggs in fathead minnows.

5 Discussion of additional studies not included in the petition published since the last amphibian SAP of 2007.

Noticeably missing from the petition references, is Kloas et al. 2009. The USEPA required that Syngenta conduct further investigations, and two experiments (U.S. and Germany) were conducted under the complete transparency of Good Laboratory Practices, and USEPA and German regulatory inspection. No impact of atrazine (0.01-100 μg/L) on metamorphosis or gonadal development was detected. The USEPA concluded “that atrazine does not adversely affect amphibian gonadal development”. A panel of independent expert scientists convened by the USEPA agreed that “reproductive fitness (sex ratio, intersex condition) were unaffected” and “there is

currently no available proof for the hypothesis regarding the purported action of atrazine on the induction of aromatase"

Four additional amphibian publications are noticeably missing from the summaries by Hayes or Rohr, presumably because they do not support the claims of the petition. LaFiandra et al (2008) exposed North American treefrogs (*Hyla verisicolor*) to 20 and 200 *µ*g/L atrazine and reported no effects on sex ratio or gonadal development. Likewise, Storrs and Semlitsch (2008) exposed three North American species (*Bufo americanus*, *Hyla versicolor*, and *Rana sphenocephala*) to atrazine (1, 3, or 30 *µ*g/L) and reported no effects on metamorphosis or ovarian development. Spolyarich et al., 2010, report no significant effects on tadpole growth, development and sex ratios of atrazine (0.1, 1, 3 and 30 *µ*g/L) in a lab study with the spotted marsh frog (*Limnodynastes tasmaniensis).* These authors concluded that atrazine did not present a significant threat to the normal development of *L. tasmaniensis* larvae in surface waters of irrigated agricultural areas. Additionally, three frog species (*L. tasmaniensis, L. fletcheri, Litoria raniformis)* were surveyed in atrazine use areas in Australia and no evidence of intersex, gonadal abnormalities, and sex ratios were found (Spolyarich et al., 2011).

Other relevant recent references have been omitted which do not support the stated effects of induction of testicular ovarian follicles or intersex in frogs by atrazine (Du Preez et al. 2009, McDaniel et al. 2008) or in reptiles (de Solla et al. 2011).

Skelly et al. (2010) conducted an analysis of the frequency of green frog (*Rana clamitans*) intersex across a range of land covers (undeveloped, agricultural, suburban and urban). Of the 11 ponds in agricultural areas 9 ponds had corn within their drainage. A total of 233 male frogs were examined for intersex (testicular oocytes). There was no evidence of a positive association with agricultural land cover and intersex. In fact the highest frequencies of intersex were positively associated with suburban and urban landscapes. While this study was not designed to identify the cause of intersex, it supports the many other studies which report no effects on amphibians from atrazine.

Relative to effects in fish, Spano et al., 2004, Tillitt et al. 2010 and Suzawa and Ingraham 2008 are cited to support statements on gonadal development, sex ratio shifts and reproductive performance in fish, while other relevant studies conducted in fish such as Bringolf et al. 2004 and USEPA 2005 which do not support the stated findings are omitted.

6 Information from mammalian toxicology studies and mode of action studies that were cited in the petition.

Table 2 addresses each of the areas of study cited in the petition for mammals. In summary, the majority of the mammalian studies cited in the petition were designed to define mode-of-action, metabolism, and toxicological endpoints. They were not designed to, nor should they be used to, predict risk to aquatic species. The studies

consistently demonstrate that effects in mammalian test systems only occur at high-dose levels that are not relevant to environmental concentrations

The three epidemiological studies in the petition have been reviewed by EPA and other regulatory bodies around the world and do not provide evidence of any potential for effects on reproductive health or cancer.

7 Conclusions

Syngenta Crop Protection, LLC provides this response to the petition of docket EPA-HQ-OPP-2011-0586. None of the information or references cited in the recent petition justify a change in the regulatory status of atrazine. Most of the studies cited in the petition have been previously considered by USEPA, and the few that are more recent do not provide compelling scientific evidence that would support this petition. The petition omitted several relevant publications from independent laboratories which indicate no effects of atrazine on amphibians.

In conclusion, USEPA should reject the petition of docket EPA-HQ-OPP-2011-0586.

8 Tables

Table 1. Studies referenced in the petition

Area of Study	Reference	Key issues in the study
Amphibian locomotion and behavior	(Rohr et al. 2003)	Significant effects on activity in salamanders were only seen at an unrealistically high exposure of 400 µg/L for 37 days. No effects observed at 4 and 40 µg/L
	(Rohr et al. 2004)	Effects in salamander behavior only seen in one year and at an unrealistically high exposure of 400 µg/L.up to 56 days. No effects observed at 4 and 40 µg/L Locomotion not specifically measured, only behavior. Use of refugia was inconsistent between years and use proportion changed between the two years of the study likely confounded by protocol changes from one year to another.
	(Rohr and Crumrine 2005)	Only one exposure of atrazine was tested (2 doses of 25 µg/L 14 days apart and 28 days study duration. . Locomotion not specifically measured, but percent of tadpoles “hiding” was reduced from 42 to 37%.
	(Rohr and Palmer 2005)	Salamander locomotion not specifically tested, only behavior (active vs. huddling). Behaviors tested 130 and 238 d after exposures to 4, 40 and 400 µg /L for 64 days. No effect at more realistic exposure of 4 µg/L.
Growth rates	(Rohr et al. 2004)	Decrease in length of salamanders at metamorphosis

		only significant at unrealistic exposures of 400 µg/L for up to 56 days. No significance at 4 and 40 µg/L.
	(Rohr et al. 2006)	Growth was not specifically measured in this study but references are made to a previously published study (Rohr et al. 2004) (above) so this is not a relevant citation.
	(Rohr et al. 2011)	Decreased mass was observed at the unrealistic exposure of 400 µg/L for 49 days, and only at 19°C, not 13°C. No effects at either 4 or 40 µg/L. Errors in the description of the preparation of the exposure solutions further call into question the robustness of the conclusions.
Cellular immunity	(Rohr et al. 2008b)	Although the trend of this relationship was significant (P = 0.047), the correlation between concentration of atrazine + DEA and melanomacrophage aggregate score gave an R^2 of 0.17 while that between phosphate and melanomacrophage aggregate score gave an R^2 of 0.32. If anything, the better correlation coefficient between phosphate and melanomacrophage aggregate score suggests that phosphate is more likely to be the key driver, not atrazine. (See additional detailed comments above in Section 4)
Trematode infections	(Rohr et al. 2008a)	Only one unrealistic exposure of atrazine (201 µg/L) for 14 days was used. Exposure of tadpoles to this concentration appeared to increase infectivity of cercaria (by about 10%) but variation between infectivity in controls between experiments was large (34 to 72%) suggesting flaws in the methodology Hypothesis generating study. The authors state "the results…should not be taken as definitive…traits that we did not quantify could affect transmission…..non-host and non-parasite species…can influence infection risk,,,,,not all hosts or life stages were tested."
	(Rohr et al. 2008b)	Although there was a significant trend between trematode infections and concentrations of atrazine + DEA[1] (P = 0.001), these were not strongly correlated (R^2 = 0.51), suggesting that the relationship was not strong. Alternative hypothesis of phosphate is a more likely explanation. (See additional detailed comments above in Section 4)
Amphibian desiccation	(Rohr and Palmer 2005)	Loss of mass in response to desiccation stress was small (<1%/h) and showed no consistent exposure-response when three salamanders were tested together. When tested singly, there was greater loss of mass (2.1-2.4%/h) but only significant at unrealistic prior exposures of 40 and 400 µg/L for 64 days. Influence of number of animals tested together > prior exposure to atrazine and realism of singlet tests is questionable.
Altered competitive interactions	(Rohr and Crumrine 2005)	Only one exposure of atrazine was tested (2 doses of 25 µg/L 14 days apart and 28 days study duration. . Indirect effects via reductions in periphytic algae were suggested as a cause of an effect on tadpoles through reduction in the availability of food. This does not

[1] DEA = desethylatrazine

		support a direct effect of atrazine on tadpoles but rather potential indirect effects.
Meta-analysis of effects	(Rohr and McCoy 2010b)	Paper presents no new data and claims to be a "qualitative" meta-analysis – all correctly conducted scientific meta-analyses should be quantitative. Overall, the study was methodologically flawed, poorly conducted, suffered from biased selection and interpretation of data, and the conclusions are not scientifically defensible.
Allegations of biased studies	(Rohr and McCoy 2010a)	This "Policy Perspective" claimed that a number of errors were made in an earlier review paper. Almost all the so called "errors" identified were in fact because of fundamental differences in approach, taking material out of context, and an apparent lack of understanding as to how toxicological studies are interpreted. No new data were presented in this paper. This paper provides no support to the claims of the petition.
Olfaction in toads	(Rohr et al. 2009)	Atrazine at an unrealistically high exposure of 201 µg/L (measured as 196 µg/L) had no effect on the olfactory detection of cercaria of *Echinostoma trivolvis* in tadpoles of *Bufo americanus*. The concentration of atrazine used was the greatest value reported from the USGS NAWQA database (years unspecified but see (Rohr et al. 2008a)). Although only one concentration was tested, it is assumed that there would be no response at lower more realistic exposures..
No increase in mortality	(Rohr et al. 2003)	No significant effects or concentration responses on hatching, survival of embryos and larvae or size were observed at exposure concentrations of 4, 40, and 400 µg/L in *Ambystoma barbouri.* Only the smallest concentration is environmentally realistic, but lack of response at the greater concentrations suggests that these observations are robust.
	(Rohr et al. 2008a)	Only one unrealistic exposure of atrazine (201 µg/L) was used in the study but no significant mortality was reported in tadpoles of *Rana clamitans* exposed for 7 d. Although only one concentration was tested, it is assumed that there would be no response at lower more realistic exposures..
	(Rohr et al. 2009)	Atrazine at an unrealistically high exposure of 201 µg/L (measured as 196 µg/L) did not cause mortality in tadpoles of *Bufo americanus*. Although only one concentration was tested, it is assumed that there would be no response at lower more realistic exposures...
Testicular lesions	(Spanó et al. 2004)	No loss of interstitial cells was observed, only an "increase in gaps in the interstitium between lobules" and this only at the highest, unrealistic concentration tested, 1000 µg/L for 21 days. Exposures to 100 and 1,000 µg/L for, did not cause effects on the relative size, number of sperm, or the relative proportions of each cell types.
Nurse or Sertoli) cells	(Tavera-Mendoza et al. 2002)	Study unreliable because of inconsistency between published papers and thesis, numbers of organisms used and lack of ability to repeat the observations in the same laboratory. This study was reviewed by 2003 and

		found to be unreliable.
Testicular tubules / germ cells	(Hayes et al. 2010)	Exposure of African clawed frogs to only one concentration of atrazine (2.5 µg/L) for 2 y after metamorphosis resulted in fewer testicular tubules with mature sperm bundles but there were no differences between exposed and control animals three years after metamorphosis. The results are thus inconsistent within the experiment. No other differences in the testes were observed. (See additional detailed comments above in Section 4)
	(Rey et al. 2009)	In juveniles caimans hatching from eggs treated with 200 µg/kg (a highly unrealistic dose), an increase in perimeter of the seminiferous tubules and a decrease in the tubular perimeter occupied by desmin positive cells was observed. No effects observed on proliferative activity, apoptosis, or cellular turnover.
Larynx and breeding glands	(Hayes et al. 2010)	The larynx size of African clawed frogs was reportedly unaffected by exposure to only one concentration of atrazine (2.5 µg/L) but it did have a different shape. The nuptial pads in exposed males were lighter in color and the breeding glands were smaller. (See additional detailed comments above in Section 4)
Female fish gonads	(Tillitt et al. 2010)	Fathead minnows were exposed to atrazine at concentrations of 0.5, 5.0, and 50 µg/L for either 14 or 30 days Two of the concentrations (0.5, 5.0) were realistic. Six types of pathological lesion were observed in the testes of the fish in the study, however, incidence was low and there were no obvious responses to concentration. Testicular ovarian follicles were observed in one fish (5 µg/L) but this fish also had granulomatous inflammation and mineralized material in the testes suggesting that this fish was atypical. (See additional detailed comments above in Section 4)
Male amphibians gonads;	(Hayes et al. 2010)	Only 10% (4 of 40) of the ZZ males exposed to the single realistic concentration (2.5 µg atrazine/L) were feminized. This is inconsistent with earlier claims by these authors of much greater rates of "feminization" at exposures as low as 0.1 µg/L. The inconsistency is not addressed or explained and leads to biological plausibility questions. No positive control was employed in this study. (See additional detailed comments above in Section 4)
	(Hayes et al. 2002)	Exposures of *Xenopus laevis* larvae to atrazine concentrations ranging from 0.1-200 µg atrazine/L throughout larval development (some but not all were realistic) resulted in gonadal abnormalities in 16-20% of the exposed animals. Since the incidence of gonadal abnormalities at each exposure-concentration was not reported, it was not possible to determine if these effects were concentration-related. . This study was reviewed in the EPA SAP 2003 and found to be inconclusive. These results have not been repeated in other laboratories..
	(Hayes et al. 2003)	This study in larvae of *Rana pipiens* used only two concentrations of atrazine (0.1 and 25 µg/L). No

		concentration response was observed with abnormalities greater at 0.1 than 25 µg/L. . This study was reviewed in the EPA SAP 2003 and found to be inconclusive. These results have not been repeated in other laboratories..
	(Hayes et al. 2006)	Larvae of *X. laevis* were exposed to concentrations of atrazine from 0.1 to 25 µg/L (25 µg/L for 65 days is not realistic)). Frequency of gonadal abnormalities in males did not show a concentration-response (author Fig. 9). These results have not been repeated in other laboratories..
Male reptiles gonads	(de Solla et al. 2006)	The petition misrepresents this reference. Eggs of snapping turtles exposed to atrazine via soil at 1-x field rate (realistic) and 10-x field rate revealed no significant effects on number of testicular ovarian follicles (TOFs) when compared to the controls. Furthermore, formulated atrazine containing a high percentage of other substances (~50%) was used for treatment, confounding interpretation of this study.
Secondary female coloration	(McCoy et al. 2008)	The reference to McCoy 2002 is incorrect as this was an unpublished conference poster. Possibly this refers to (McCoy et al. 2008) where there appeared to be a change in "coloration score" of adult *Bufo marinus* across collection sites with increasing agricultural intensity. However, there were no measurements of exposure to atrazine so there is no specific linkage.
Feminization in fish	(Suzawa and Ingraham 2008)	While the paper reported that a single replicate of zebrafish (*Danio rerio*) exposed to atrazine for 60 days at environmentally unrealistic concentrations of (21.7, 217, 2,167 µg/L) displayed a concentration dependent increase in the percentage of females and a decrease in the percentage of males, the data for sex ratios appears to be incorrect. In this study, neither the total number of zebrafish was counted nor the actual number of fish that were male or female. The numbers of fish of each sex do not add up to the number used in the study. Mortality was also not reported, which is especially important in the high dose group (2,167 µg/L) because this group was exposed at a concentration in excess of the established early-life-stage 35 day- LC_{50} of 890 µg/L (Gorge and Nagel, 1990). Therefore, these data are difficult to interpret. This study has been repeated twice at the same concentrations by another laboratory and no effects on sex ratio or gonadal abnormalities were observed (Corvi et al. 2011).
Feminization in amphibians	(Oka et al. 2008)	This study reported an effect on sex ratio in *X. laevis* at concentrations with significantly more females at 10 and 100 µg/L but not at 0.1 and 1 µg/L. Statistical significance was likely an artifact of a high percentage of males in the control (61%). Comparison should have been to 50% as reported in the literature. In fact the authors state the authors concluded that "higher female ratios in atrazine exposure groups in the present study were not caused by estrogenic action of atrazine."

	(Langlois et al., 2009)	This was a study of the effects of atrazine applied at two realistic concentrations (0.2 and 3.7 µg/L) to *R. pipiens* in microcosms. the authors reported a significantly altered sex ratio in tadpoles exposed to 3.7 µg/L, with 42% male as compared to the 56% in 0.2 µg/L and 62% in the control. Reported effects likely due to variation in sex ratios at the start of the study. The comparison should have been to the normal ratio of 50% as reported by others including co-workers in the same laboratory. The positive EE2 control failed to confirm sensitivity of the system to feminization. Interpretation of this study is therefore limited. (See additional detailed comments above in Section 4)
Feminization in reptiles	(Willingham 2005)	Eggs of *Trachemys scripta elegans* were treated with 0.5 µg/kg atrazine in solvent and incubated at 26 and 29.2°C. The controls incubated at 26°C produced 60% males and those at 29.2°C 48% males. Eggs treated with atrazine and incubated at 26°C produced 54% males and 32% at 29.2°C. The decrease in males in atrazine-treated eggs incubated at 29.2°C was significantly less than the control incubated at 26°C but the scientifically appropriate comparison to the control at 29.2°C was not made. Thus, there was no significant effect of atrazine at 26°C and any potential effect at 29.2°C is unknown. A major fault in this study lies in the data comparisons made.
Androgens in fish;	(Moore and Waring 1998)	Salmon (*Salmo salar*) were exposed to nominal realistic concentrations of atrazine at 0, 0.5, 5, 10 and 20 µg/L for 5 d and then challenged with urine collected from ovulated female salmon. Consistent with a suppression of olfactory responses to pheromones by atrazine, an exposure-dependent decrease in concentrations of plasma steroids (17,20β -dihydroxy-4-pregnen-3-one, testosterone, and 11-ketotestosterone) was observed. A concentration-response was observed although the statistical comparison was incorrect,.
Androgens in amphibians	(Hayes et al. 2010)	In contrast to earlier work (Hayes et al. 2002), the authors reported testosterone concentrations in plasma of control ZZ males ranging from 0.0 to 40 ng/mL and in atrazine-treated ZZ males from 0.0 to 25 ng/mL. This inconsistency with previous Hayes et al publications is not explained. It is surprising that some basal activity was not measured in all males. (See additional detailed comments above in Section 4)
	(Hayes et al. 2002)	In this experiment, four adult *X. laevis* were exposed to an unrealistic concentration of 25 µg atrazine/L for 46 days. The concentration of testosterone in the plasma of these animals was significantly depressed. This study was reviewed in the EPA SAP 2003 and found to be inconclusive. These results have not been repeated in other laboratories..
	(McCoy et al. 2008)	The reference to McKoy 2002 is incorrect as this was an unpublished conference poster. Possibly this refers to (McCoy et al. 2008) where there reportedly was a significantly lower concentration of testosterone in plasma of male *B. marinus* from agricultural vs. non-

		agricultural areas. However, there were no measurements of exposure to atrazine so there is no specific linkage.
Androgens in reptiles;	(Rey et al. 2009)	No significant decrease in testosterone concentrations were observed in serum of juveniles hatching from eggs of *Caiman latirostris* treated with 200 µg/kg (an unrealistic concentration). Solvent delivery of atrazine into the egg is not realistic. This is an incorrect interpretation of the paper by Hayes.
Aromatase or estrogen production in fish;	(Spanó et al. 2004)	Exposure of goldfish to an unrealistic concentration of 1000 atrazine µg/L for 21 days (but not 100 µg/L) caused a decrease in testosterone and 11-keto-testosterone concentrations in plasma and increased plasma estradiol concentrations in males. However, there were no changes in the concentration of vitellogenin in plasma, which is an estradiol-dependent response. Thus, concentration changes reported in plasma were either transitory, in error, or not sufficiently great to cause adverse effects on reproductive function.
	(Suzawa and Ingraham 2008)	This study reported significant but small increases in *Cyp19A1* gene expression in ovaries of zebrafish (*Danio rerio*) exposed to atrazine for 3 days at concentrations of 2.17 µg/L (realistic) and 21.7 µg/L with a maximum increase in expression of 5.5-fold at 2,171 µg/L (unrealistic). However, interpretation of these data must be considered carefully as the data are from a single treatment tank with only three fish. Therefore, the biological significance cannot be ascertained with any certainty. Additionally, the authors did not address the inconsistencies with other studies, such as Kazeto et al. 2004 or Nadzialek et al. 2008.
	(Moore and Waring 1998)	Aromatase activity and concentrations of estrogen were not measured in this study. This claim is incorrect.
Aromatase in amphibians	(Hayes et al. 2010)	There was no increase in the activity of aromatase in ZZ males exposed to a single realistic concentration (2.5 µg atrazine/L). Also, there was no measurement of estradiol, which would have been expected to increase if aromatase was induced.
Male reproductive behavior in fish;	(Moore and Waring 1998)	The olfactory epithelium of salmon (*S. salar*) was exposed to nominal realistic concentrations of atrazine at 0, 0.5, 5, 10 and 20 µg/L for 5 d and the electrophysiological response to prostaglandin $F_{2\alpha}$ measured. Response decreased with increasing concentrations and significant differences at 2 µg/L. This study measured effects on sensory organs *in vitro* and not a behavioral response as claimed.
Reproductive behavior in amphibians;	(Hayes et al. 2010)	Behavioral impairment of reproduction in the ZZ males exposed to a single realistic concentration of atrazine (2.5 µg /L) was reported. However, method details are limited and only four mating trials were conducted. No positive control was employed in this study. No evidence of reproductive impairment exist in atrazine use areas in Africa were this species occurs. as the ZZ males used in this study are not found in nature and were produced using large exposures to estradiol, the

		results cannot be extrapolated to normal frogs.
Sperm production and fertility in fish;	(Moore and Waring 1998)	This paper did not report production of sperm or measures of fertility. Hayes is possibly referring to the (Moore and Lower 2001) where expressible milt was measured and showed no concentration response to 0.5 and 2 µg/L atrazine. There was however, a reduction from control fish exposed only to prostaglandin $F_{2\alpha}$, presumably through the olfactory mechanism. Fertility was not measured.
Fertility in amphibians;	(Hayes et al. 2010)	The ZZ males exposed to a single realistic concentration of atrazine (2.5 µg /L) did produce significantly fewer fertilized eggs. However, as the ZZ males used in this study are not found in nature and were produced using large exposures to estradiol, the results cannot be extrapolated to normal frogs.

Table 2: Summary of *In vivo* and *In vitro* Mammalian studies

Area of Study	Reference	Key issues in the study
Testicular tubules / germ cells	(Kniewald et al. 2000)	Male Fischer rats were dosed twice a week for 60 days with either 60 or 120 mg/kg body weight via interperitoneal injections. An approximate 15% body weight loss was observed in both the 60 and 120 mg/kg dose group indicating that the maximum tolerated dose was exceeded in this study. Results were inconsistent with shorter term studies conducted in the same laboratory as well as other studies using a more appropriate route of administration (oral), all of which did not have any effects on male fertility or histological effects in male testes. All concentrations were above the water solubility limit of atrazine and well above environmental concentrations.
	(Victor-Costa et al. 2010)	Wistar male rats were exposed to a daily gavage doses at 50, 200, and 300 mg/kg/day, all concentrations were above the water solubility limit of atrazine and well above environmental concentrations.
Androgens	(Friedmann 2002)	Leydig cells from 49 day old Sprague-Dawley rats were isolated and incubated in cell cultures with atrazine at a concentration of 50 ppm in 0.2% DMSO. All concentrations were above the water solubility limit of atrazine and well above environmental concentrations.
	(Gojmerac and Kniewald 1989)	This is a metabolism study and androgen levels were not measured.
	(Kniewald et al. 1995)	All concentrations were above the water solubility limit of atrazine and well above environmental concentrations.
Aromatase	(Suzawa and Ingraham 2008)	This study was only done in a mammalian cell line (JEG-3) *in vitro* with similar results to those of Sanderson et al. (2000). Not relevant to *in vivo* responses.
	(Sanderson et al.	This study was done in H295R and JEG-3 cells *in vitro*

	2000)	and induction was observed at concentrations of 43 µg/L and higher. This response is not a relevant to *in vivo* effects.
	(Sanderson et al. 2001)	This study showed that aromatase could also be induced *in vitro* in MCF-7 cells but that there was no induction of vitellogenin production in male carp hepatocytes; nor antagonism of the induction of vitellogenin by 100 nM 17β-estradiol at an atrazine concentration of 6500 µg/L (highly unrealistic). This demonstrated lack of relevance in whole tissues.
	(Sanderson et al. 2002)	This study was done in H295R cells. Similar results to those in previous work (Sanderson et al. 2000). This response is not a relevant to *in vivo* effects.
	(Heneweer et al. 2004)	This *in vitro* study repeated the observations on induction of aromatase in H295R but showed that rat Leydig tumor cell line R2C cells were unresponsive up to an extremely unrealistic concentration of 21,700 µg/L. Thus, not all cells are responsive.
	(Fan et al. 2007a) and (Fan et al. 2007b)	These papers reported results of mechanistic studies, which showed that, in cell cultures, steroidogenic factor 1 (SF-1) is involved in the process of induction of aromatase. Not relevant to whole-animals.
	(Eldridge et al. 1999a)	Mode of action research studies in Sprague Dawley rats at concentrations well above environmental concentrations.
Reproduction	(Kniewald et al. 2008)	Abstract. An assessment cannot be made as there are no data presented.
	(Peruzović et al. 1995)	The primary purpose of this study was to determine the effects of high doses of atrazine on the estrus cycle, ability to copulation and conception, course of pregnancy and delivery. None of these parameters were affected by atrazine exposure for six days at high doses of 120 mg/kg/day. In addition, litter size, pup survival, and body weight of the offsprings were also unaffected. The only positive finding in this study was body weight reduction in treated females and slight increase in spontaneous activity in female offsprings.
Sperm production and fertility	(Kniewald et al. 2008)	Abstract. An assessment of the science cannot be made as there are no data presented.
	(Abarikwu et al. 2008 and 2010)	Wistar rats were exposed, orally to concentrations in excess of the water solubility limit of atrazine (note - we could not locate the 2008 citation).
	(Šimić, B., et al., eds. 2001)	All concentrations were above the water solubility limit of atrazine and well above environmental concentrations.
	(Pintér et al. 1980)	Pinter et. al., 1980 conducted a study on Fischer-344 rats fed atrazine at dietary levels of 375 or 750 ppm for a life time. EPA's Health Effects Division (HED) reviewed this study. In contract to the conclusions drawn by the author, the HED reviewer in a memo dated 1/29/99 conclude: 1) There was increased survival among females in the atrazine treated groups compared to the controls.

		2) When considered separately there is not a statistically significant increase in either leukemias or lymphomas in either sex. 3) The increases seen in uterine carcinomas in the atrazine treated females are likely a false positive resulting from age-related (not treatment related) increase in mammary tumor incidence. 4) The increase in male benign mammary tumors appears to be due to the increased survival of the males in the high dose group.
	(Stanko et al. 2010)	Study design questionable: 1) Dose-response data for individual atrazine metabolite components in the mixture were not performed. 2) No data on chemical analyses of the mixture were provided to determine if the concentrations of the metabolites was constant as a function of time. 3) Incidence and severity of findings subsided with time despite continued dosing. 4) Hormones in these animals were generally not different among exposure groups and were within the normal range. 5) Animals excluded from the hormonal analyses were not excluded from other analysis. EPA reviewed this study in July 2011 and questioned study design.
Hormone – dependent disease processes	(Eldridge et al. 1999a)	Mode of action research studies in Sprague Dawley rats at concentrations well above environmental concentrations.
	(Ueda et al. 2005)	This study showed that atrazine at doses up to 500 ppm in the diet does not promote DMBA-induced tumors in ovariectomized animals. EPA reviewed this study in April 2010.
Mammary gland	(Eldridge et al. 1994)	Study concludes that the risk of atrazine-related mammary tumors in humans would be quite low because of the unique response of the Sprague-Dawley rat estrous cycle, the nature of stimulated mammary tumors in rats, and extremely high dose levels and concentrations required for atrazine to express activity in vivo and in vitro. The mode of action for mammary tumors in the Sprague Dawley rat has been determined by regulatory agencies around the world to be not relevant to humans.
	(Eldridge et al. 1999b)	The high-dose exposures as well as differences in neuroendocrine senescence of Sprague Dawley rats support a lack of relevance to humans.
	(Stevens et al. 1994)	Mode of action research studies in Sprague Dawley rats at concentrations well above environmental concentrations. These results support a hypothesis that an earlier onset and/or increased incidence of mammary tumors is unique to the Sprague-Dawley female rat and occurs above a high threshold dose.

	(Tennant et al. 1994)	Mode of action research studies in Sprague Dawley rats at concentrations well above environmental concentrations.
	(Wetzel et al. 1994)	Mode of action research studies in Sprague Dawley rats at concentrations well above environmental concentrations. These results support the hypothesis that an earlier onset and/or increased incidence of mammary tumors is unique to the Sprague-Dawley female rat and not relevant to humans.

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