

June 1, 2010

Dr. Ralph J. Cicerone, President
National Academy of Sciences
500 Fifth St., N.W.
Washington, D.C. 20001

Dr. Randy Schekman, PNAS Editor in Chief
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Dear Sirs;

The Proceedings of the National Academy of Sciences has a long history of publishing peer reviewed scientific papers of a high standard. It is for this reason that I, on behalf of Syngenta, would like to express our disappointment that on March 1, 2010 the PNAS published ahead of print *Atrazine induces complete feminization and chemical castration in male African clawed frogs (Xenopus laevis)* by Tyrone B. Hayes, Vicky Khoury, Anne Narayan, Mariam Nazir, Andrew Park, Travis Brown, Lillian Adame, Elton Chan, Daniel Buchholz, Theresa Stueve, and Sherrie Gallipeau. Vol. 107 no. 10 4612-4617. www.pnas.org/cgi/doi/10.1073/pnas.0909519107

The paper contends that long term exposure to low levels of atrazine causes partial or complete feminization of frogs. The paper has multiple methodological weaknesses which include serious design and reporting limitations. First, the study includes only one dose level, and while atrazine water analyses were reportedly conducted, no data are provided. In addition, sampling and statistical methods are not clearly reported in many cases, making evaluation of the study difficult. Of most concern, there is no positive control which is necessary for system validation and comparison of effects. Inclusion of an estradiol positive control would have allowed testing of the biological plausibility of the key effects reported in this study.

Additionally, the authors inappropriately extrapolate these results to wild frog populations. The study was conducted using ZZ males which were the offspring of normal males mating with genetic males which were estradiol-induced, sex-reversed, phenotypic females. While this technique has advantages when focused on male only effects, it also brings a degree of uncertainty to the interpretation of risk in the wild. In fact, Dr. Hayes has previously been critical of other researchers for the use of ZZ males, when their use failed to produce effects which supported his previous findings (Renner, 2008).

The authors misrepresent conclusions from publications of some other scientists to support their own statements. For example, when referring to Carr, et al. (2003) the authors continue to misrepresent their findings and do not acknowledge that these findings were subsequently corrected by Carr in Solomon, et al. (2008). Using data from Oka et al. (2008), the authors create a graph and state that atrazine alters sex ratios, yet neglect to report the statistical analyses conducted by Oka, et al. (2008) or their reported conclusion 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."

In contrast, in an article in *Environmental Science and Technology* (Renner, 2008), Hayes previously criticized this same Oka publication for failing to duplicate his findings, stating they "did not get effects in their all-male population because they are a different population, and more importantly, they did not test the effective doses with the all-male animals".

To support their hypothesis, Hayes et al. (2010) misrepresent Reeder, et al. (1998) by citing that feminization and demasculinization of amphibians is "directly correlated with atrazine contamination in the wild". In fact, Reeder et al. (1998) report no significant intersex differences in cricket frogs although they report an association "approaching significance". Further, they recognize that "atrazine contaminated sites may represent areas of great agricultural impact, potentially involving other chemical, physical, and biotic factors" Further, while misrepresenting Reeder et al. (1998) to support their hypothesis, the authors do not note that Reeder et al. (2005) found that the greatest incidence of intersex in cricket frogs occurred before the introduction of atrazine in agriculture.

This is the latest in a series of publications by Dr. Hayes that purport to show that atrazine alters the sexual development of amphibians. The biological findings in the 2010 publication appear inconsistent with previous publications by Dr. Hayes as explained in detail in the attachment to this letter. Finally, the previous publications have significant flaws. Over several years, a range of regulatory bodies have disagreed with or discredited the Hayes hypothesis and its significance:

- The 2002 PNAS publication, *Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses*. T. B. Hayes, M. Lee, M. Mendoza, N. Noriega, A.A. Stuart. (PNAS USA 99 5476 – 5480) was critiqued by the UK rapporteur in an EU regulatory review: "In general, little data and insufficient details of statistical analyses are given in the paper. The work reported in the paper was apparently carried out with little regard for assessment of causality. Hence, the paper grossly overstates the potential effects on frogs and changes in populations based on the data given" (UK 2003).
- Also, Hayes' published findings were twice reviewed by USEPA scientists (USEPA 2003, USEPA 2007a) and considered by a Scientific Advisory Panel (USEPA 2007b) and the hypothesis was refuted.
- A similar scientific review of atrazine's potential impacts on amphibians, which included Hayes publications, by the Australian authorities concluded "that current data indicate that it is unlikely that atrazine is impacting adversely on Australian amphibian populations at current levels of exposure" (APVMA, 2008).

Given that these previously purported 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.

Atrazine has become the target of many high profile media pieces surrounding this publication in which the findings of the study are represented as fact. Syngenta continues to support the regulation of atrazine based on sound science. Publications like PNAS need to provide forums for objective scientific discussion and rigorous review.

Syngenta is disappointed and concerned that a publication with so many obvious weaknesses could achieve publication in such a reputable scientific journal. We are concerned that the editorial process used for this publication was neither objective nor independent, and that the important rigor of the scientific review was absent. Can the Academy and PNAS confirm that the usual objective PNAS editorial standards were applied in this case?



In addition, in the PNAS *Information for Authors* document, one of the journal policies listed relates to materials and data availability. It states, "to allow others to replicate and build on work published in PNAS, authors must make materials, data and associated protocols available to readers. Authors must disclose upon submission of the manuscript any restrictions on the availability of material or information. Data not shown and personal communications cannot be used to support claims in the work. Authors are encouraged to use Supporting Information to show all necessary data. Authors are encouraged to deposit as much of their data as possible in publically accessible databases. Such deposition may facilitate access to data during the refereeing process and post-publication." Syngenta has looked at the PNAS website and cannot find data needed to replicate and build on this work. We would ask PNAS/NAS to ensure that these materials, data and associated protocols are made publicly available as required by the PNAS and further, that Dr. Hayes is given a deadline for posting of these materials, data and associated protocols.

Syngenta has prepared and will submit a rebuttal to this paper to appropriate regulatory agencies. We also attach an abbreviated version of this rebuttal for your information.

Again, I would ask for a response from the Academy confirming that PNAS editorial standards were applied in the review of Dr. Hayes' paper. I would also request that PNAS notify Syngenta when and where Dr. Hayes will place pertinent materials, data and associated protocols in a publically available database. Further, I would ask the PNAS to assure that the quality and quantity of the materials and data made available is sufficient to allow study replication.

Sincerely,

A handwritten signature in black ink, appearing to read "Peter Hertl". The signature is written in a cursive, flowing style.

Dr. Peter Hertl
Head, Global Product Safety

References Cited

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Hayes TB, Khoury V, Narayan A, Nazir M, Park A, Brown T, Adame L, Chan E, Daniel Buchholz D, Stueve T, Gallipeau S. Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). *Pro Natl Acad Sci USA* 107:4612-4617, www.pnas.org/cgi/doi/10.1073/pnas.0909519107

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USEPA. 2007b. FIFRA Scientific Advisory Panel Meeting, October 9-11, A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding: The Potential for Atrazine to Affect Amphibian Gonadal Development. Arlington, VA, USA: United States Environmental Protection Agency. No. FIFRA SAP Minutes No 2007-08. 32 p. <http://www.epa.gov/scipoly/SAP/meetings/2007/october/finalminutes.pdf>

Technical Evaluation of Scientific Publications

Syngenta Crop Protection

The following is a critical review of the scientific publication:

Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*)

Tyrone B. Hayes^a, Vicky Khoury^a, Anne Narayan^a, Mariam Nazir^a, Andrew Park^a, Travis Brown^a, Lillian Adame^a, Elton Chan^a, Daniel Buchholz^b, Theresa Stueve^a, and Sherrie Gallipeau^a

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PNAS Early Edition

Abstract (Hayes et al. 2010)

The herbicide atrazine is one of the most commonly applied pesticides in the world. As a result, atrazine is the most commonly detected pesticide contaminant of ground, surface, and drinking water. Atrazine is also a potent endocrine disruptor that is active at low, ecologically relevant concentrations. Previous studies showed that atrazine adversely affects amphibian larval development. The present study demonstrates the reproductive consequences of atrazine exposure in adult amphibians. Atrazine exposed males were both demasculinized (chemically castrated) and completely feminized as adults. Ten percent of the exposed genetic males developed into functional females that copulated with unexposed males and produced viable eggs. Atrazine exposed males suffered from depressed testosterone, decreased breeding gland size, demasculinized/feminized laryngeal development, suppressed mating behavior, reduced spermatogenesis, and decreased fertility. These data are consistent with effects of atrazine observed in other vertebrate classes. The present findings exemplify the role that atrazine and other endocrine-disrupting pesticides likely play in global amphibian declines.

Syngenta Executive Summary

- This publication is the latest in a series authored by Dr. Tyrone Hayes that purports to demonstrate that atrazine alters the sexual development of amphibians. 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 refuted. After a similar scientific review including the Hayes publications, the Australian authorities concluded "that current data indicate that it is unlikely that atrazine is impacting adversely on Australian amphibian populations at current levels of exposure (APVMA 2008). In contrast to the Hayes publications, a new publication (Spolyarich et al., 2010) which was not available at the time of Hayes et al. (2010), report no effects of atrazine on sexual development in amphibians (spotted marsh frog). Given that these refuted effects of atrazine on sexual development are the biological basis and foundation of the current publication, the new effects reported in Hayes et al. (2010) are questionable.
- The biological plausibility of the purported effects in Hayes et al. (2010) has not been demonstrated, although this could have been investigated using an estradiol positive control. Estradiol is the only chemical reported to cause complete feminization in *Xenopus laevis*, such as here described with atrazine.
- Biological findings (eg. measures of testosterone, laryngeal muscle size, and intersex) are not consistent with previous publications by Hayes (see below).
- This publication reports on a study which 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. While atrazine water analyses were reportedly conducted, no data are provided.
- The publication suffers from a lack of clarity concerning design, sampling and statistical methods as well as results, thus hindering a complete evaluation of the study.
- The study was conducted using ZZ males which were the offspring of normal males mating with genetic males which were estradiol-induced, sex-reversed, phenotypic females. This adds a degree of uncertainty relative to the extrapolation of these findings to natural frog populations. In fact, Hayes criticized the use of a ZZ male population by Oka, et al. (2008) in which effects of atrazine were not observed (Renner 2008). Hayes et al., (2010) state that in this study the ZZ males (n = 8, control or treated) did not express aromatase. It is unexpected that males do not express any aromatase and inconsistent with many other studies, including the ZZ males in Oka et al (2008).
- To support their findings, the authors appear to misrepresent the conclusions from some of the publications of other scientists. Publications not in support of their

hypothesis are not considered or are inaccurately criticized. Many of the studies cited to support their work (including studies conducted by the lead author) have not withstood rigorous scientific review by regulatory agencies and independent science panels.

- The design of the study and lack of detail included in the paper and the Supplemental Information (SI) prevents a scientific interpretation as to any relevance to the effects of atrazine on amphibians.

Study Methods and Design (Hayes et al., 2010)

The paper and the Supplemental Information (SI) describe the results of exposures to atrazine on sexual development and reproduction in ZZ male *Xenopus laevis* frogs. Treatments included one atrazine dose level (nominally 2.5 µg/L) and an EtOH solvent control (0.0003%). Analysis of atrazine was reportedly conducted with Abraxis ELISA, however, no results are provided for evaluation. Documentation of chemical analysis of controls is not provided. Animals were reared in 10% Holtfreter's solution, and Holtfreter's and atrazine solutions were renewed every 72 hours. Initial exposure began "from hatching" and continued until Stage 66. Information is not provided on hatching success, early stage mortality which normally occurs, or the process of selecting newly hatched frogs for the study.

The authors indicate that of the initial 270 frogs/treatment, 3 replicates (90 frogs) from each treatment were sacrificed at Stage 66 metamorphosis, "for analysis not reported here". The remaining 180 frogs from 6 tanks were mixed, and 120 frogs per treatment were distributed into 3 tanks (40 frogs/tank) and reared to sexual maturity (2 or 3 years). Of the 120 frogs, 34 were reportedly used for fertility and mate choice studies. The authors report 5 mating trials in SI, Fig S1, however report the results for 4 trials only. Method details for these mating trials are largely absent. Eighteen (18) frogs were sampled 2 years after metamorphosis for testicular examination and 10 frogs similarly were examined 3 years after metamorphosis. Sample sizes for examination of nuptial pads (presumed n=14 from Fig 3) and larynx morphology (n=11) are reported, however, it is not clear which frogs were used for multiple metrics and why sample sizes are unequal between metrics. The fate of the remaining frogs is not provided other than "The remaining animals were used for studies not described here". This raises questions about the selection process of the experimental animals and scientifically appropriate randomization within the study.

Detailed Comments on Hayes et al. (2010)

- There are a number of experimental weaknesses in the design of this study. Typically, studies used to assess the effects of substances on animal test systems are conducted at more than one concentration. This allows characterization of dose or concentration responses. Observation of a concentration-response is a key factor in assigning causality and coherence of response; therefore results from the single dose used in this experiment are not valid for this purpose.
- Lack of a positive control such as estradiol (E2) or ethinylestradiol (EE2), used in numerous other studies, is another significant study design weakness. E2 has been shown to have an EC50 for feminization of males of ~0.2 µg/L (Wolf et al., 2010) and in a related species, *X. tropicalis*, an EC50 for feminization by EE2 has been demonstrated at ~1.8 ng EE2/L (Gyllenhammar et al., 2009). Complete feminization of male *X. laevis* such as attributed by Hayes et al. (2010) to atrazine has been well documented for E2 (Witschi, 1930; Oka et al., 2008). Use of a positive control is essential in determining validity and sensitivity of the test system. The lack of a positive control in Hayes et al., (2010) study puts the study results in question and limits comparison with reported effects from other studies.
- The methods (as detailed in the SI) are presented in insufficient detail. The basic husbandry of the frogs is not clearly described. For instance, it is not apparent if the larvae were exposed in plastic containers as in Hayes et al. (2002a), which could potentially leach plasticizers (which may be biologically active, Oehlmann et al., 2009). The adult frogs were held in fiberglass containers which could have adsorbed and then desorbed substances that may confound the results. There were no chemical analyses of the water for chemicals other than atrazine (no data given) and no receptor-mediated analyses (YES and YAS) were conducted to determine the absence of estrogenic and androgenic substances.
- Selection of animals within the study is not adequately described in the SI. The authors state that “At metamorphosis, three replicates from each treatment were killed for analyses not reported here. The remaining animals from each treatment were mixed and apportioned into three replicates in tanks (79 × 91 × 30 cm).” While some of the replicated tanks of the larvae were used in other studies, the apportionment of the remaining animals is not sufficiently described, particularly as this pertains to scientifically valid randomization. The use of the animals in the “other studies” is not clearly described.
- The SI also states that “For analyses in the present study, animals were divided into 38 tanks (46 × 25 × 20 cm) and reared singly (for fertilization experiments) or in groups of

four (four breeding competitions) as described above”. The selection process of the animals for the “present study” is unclear, and there are no details of the randomization procedures. From Fig S1, 18 tanks were used for each treatment (0 and 2.5 µg atrazine/L). Fourteen (9+5) of these tanks contained single animals and 5 contained 4 each for a total of 14+20 = 34 animals. The source of the 40 animals reported as fully or partially feminized males used in the study is not apparent.

- There is a lack of clarity on pooling of data for statistical analysis. In several of the analyses, it is not clear if the data from animals sampled in 2007 and 2008 were pooled. However, there were differences between the animals sampled in 2007 and those in 2008. For example, it is stated that there were differences in the “relative number of testicular tubules with mature sperm bundles” in animals (n=18) sampled in 2007 but not in animals (n=10) in 2008. Figure 5 (Hayes et al., 2010) indicates that percent fertility data from 2007 and 2008 were pooled for analysis. Given the observed difference in testicular sperm bundles, data for percent fertility for each year should have been analyzed separately.
- While ZZ males may assist with an understanding of determination of sex and other processes related to reproductive development in this species, their use introduces some uncertainty relative to extrapolation of risk to wild populations. The ZZ males used in this study are not identical to those of this species in nature. While they are genetic males, they are produced by manipulation of sexual development by exposures to estradiol and virtually nothing is known about epigenetic effects, which would be different from those in males produced in the normal manner. These epigenetic factors are important as some of the processes involved in metabolism of sterols are associated with the mitochondria which would normally be inherited directly from the mother and not via a male. It is known that patterns of sterility in ZZ and ZW frogs in the *Xenopus* genus are atypical (Malone and Michalak 2008)..

Interestingly, in an apparent acknowledgment of the uncertainty associated with the use of ZZ males for such testing, and while commenting on a publication (Oka et al., 2008) in which effects similar to those reported in the publication were not observed with atrazine, Hayes stated “ [Iguchi and colleagues] did not get effects in their all-male population because they are a different population, and more importantly, they did not test the effective doses with the all-male animals,” (Renner, 2008). Concentrations (doses were not measured) in Oka et al. (2008) were 0.1 and 1.0 µg/L, which are the same as those reportedly previously by Hayes to cause effects (Hayes et al., 2002; Hayes et al., 2003).

- The authors state that in this study the ZZ males (n = 8, control or treated) did not express aromatase. It is unexpected that males do not express any aromatase. Many studies have

demonstrated that the testis of *Xenopus*, both during development and in the adult, exhibit aromatase activity and the expression of Cyp19 gene (Hecker et al. 2005b, Park et al. 2006, Oka et al. 2008). Oka et al. (2008) also employed ZZ males in their study and demonstrated aromatase expression in these animals. This suggests inappropriate analysis.

- Hayes et al., (2010) state that “In fact, more than a half million pounds of atrazine are precipitated in rainfall each year in the United States “(2). However, this statement is not found in the reference cited (Thurman and Cromwell, 2000). Thurman and Cromwell (2000) measured concentrations of atrazine in rainwater and surface waters at Isle Royale National Park in Lake Superior in 3 years. One can approximate the Hayes et al., (2010) figure of “half million pounds” by multiplying the entire area of the US ($9.63 \times 10^{12} \text{ m}^2$) by the 3 year average concentration reported by Thurman and Cromwell (23.7 $\mu\text{g}/\text{square m}/\text{year}$) and converting to pounds (503,000 lbs).

However, this incorrectly assumes all of the U.S. (including upwind, downwind, use and no-use areas) is represented by the Isle Royale Park relative to likely occurrence of atrazine in rainfall. This approach obviously grossly overestimates the amounts precipitated nationwide. Also, annual values reported by Thurman and Cromwell (above) were for atrazine, DEA, DIA, and cyanazine combined. These other triazines represented between 31 and 58% of the mass, with cyanazine alone accounting for between 6% and 52% of the total mass. These data simply do not support the statements in Hayes et al. (2010).

- The Hayes et al., (2010) claim that atrazine is a potent endocrine disruptor is based on a number of poorly conducted studies in:

Fish: The citations to effects on fish include (Moore and Waring, 1998) were based on responses to the priming effect of ovulated female salmon urine, changes in androgen secretion, and changes in steroid concentrations in the bile, but several hormones were omitted from the analysis. Hayes et al., (2010) do not mention the results of a 21-d reproduction bioassay where fish (*Pimephales promelas*) were exposed to atrazine at measured concentrations of 25 and 224 $\mu\text{g}/\text{L}$ and showed no treatment-related effects on estradiol or testosterone in females or testosterone and 11-ketotestosterone in males (USEPA 2005). A similar study by Bringolf et al. (2004) utilized a static-renewal protocol (25% per day), two concentrations of atrazine (5 and 50 $\mu\text{g}/\text{L}$) and estradiol as a positive control. No effects of atrazine on a number of parameters including numbers of eggs produced were reported, however, estradiol did cause a reduction in numbers of eggs as well as other responses. Furthermore, four fish tests have been conducted with atrazine in which fish were exposed for throughout the life cycle (274-450 days) and at

high atrazine concentrations (of up to 2000 µg/L). Exposures were from an early life stage (embryo/larval/juvenile) throughout development and reproductive stages until a pre-determined number of spawns occurred (Macek et al., 1976; Dionne 1992). No effects on reproduction from these long-term (274-450 days) and high exposure studies (up to 2000 µg/L) were observed. The study on zebrafish (Suzawa and Ingraham, 2008) mentioned in Hayes et al., (2010) reported effects on sex ratio that are not possible to interpret from the data presented. In author Figure 2A (Suzawa and Ingraham, 2008), exposure of fish to 217 µg atrazine/L is stated to increase the number of females by 400% (four-fold). Unless sex ratio in the fish deviated greatly from the nominal 50:50 at study initiation, this would mean that the number of females increased from approximately half of the total of 15 in the beaker to 30 fish ($15/2 \times 4$). Clearly, there is an error in the data or the description, and in either case the data are not usable.

Amphibians: Carr et al. (2003), did not report potent endocrine disruption. The study by Hayes et al. (2002, 2003) had serious design flaws and results did not show a monotonic response to concentrations of atrazine. The cited paper by Hayes et al. (2006) was flawed by poor survival in treated animals and by the inclusion of data from previous studies that were claimed to have been from studies conducted at different concentrations of exposure. Because of incomplete descriptions in the methods and inconsistencies in the data, the results cited in the papers by Tavera-Mendoza et al. (2002a, 2002b) and the thesis on which they are based (2001) cannot be used to support the hypothesis that atrazine adversely effects gonadal development in frogs. These studies were all examined previously by EPA (USEPA 2003; USEPA 2007a; UAEPA 2007b) and found not to support the suggestion that atrazine is an endocrine disruptor in amphibians.

Cell lines: The citations used to support effects in cell lines are based on studies carried out on transformed cells at high exposures and in the absence of metabolism found in intact animals. Fan et al. (2007b, 2007a) used a mammalian cell line transfected with multiple copies of the ArPII promoter, high copy levels of SF-1, and exposed to high concentrations of atrazine or simazine (often > 2,000 µg/L and up to 21,500 µg/L). Even in these high exposure cases, expression of *CYP19* mRNA was often just ~1.5 fold greater than the control levels. Given the complexity of this artificial cell system and the very high and environmentally irrelevant concentrations of the triazines required to mediate these effects, the significance of these observations in whole organisms and tissues other than these cell lines is questionable. Similarly, the observations of induction of aromatase in other cell lines (Sanderson et al., 2000, Sanderson et al., 2001), were reported at high concentrations and not in the rat R2C cell line (Heneweer et al., 2004) or in tissues from fish (Sanderson et al., 2001).

Reptiles: Contrary to the authors' claim "that atrazine is a potent endocrine disruptor

active in the ppb (parts per billion) ranges” exposures to atrazine did not result in any adverse effects on developing alligators (*Alligator mississippiensis*) (Crain et al., 1997; Spiteri et al., 1999). Crain et al. (1997) reported that eggs were dosed with atrazine from 140 µg/L to 14000 µg/L in ethanol solutions to transport the compound into the eggs. They stated that “Among the other eggs incubated at a male temperature, both E₂ and atrazine-treated hatchlings appeared to have elevated aromatase activity.” However doses were not statistically different and aromatase activities were not statically different from control males or control females. Importantly, no effects on sex reversal were observed. In a subsequent paper by Crain and coauthors (Spiteri et al., 1999) of similar design the authors concluded that atrazine did not affect sexual development in *A. mississippiensis* hatchlings and was not responsible for effects on sexual development observed in wild *A. mississippiensis*. Similarly, but not cited, inconsistent results were seen in cell-lines from turtles (Keller and McClellan-Green, 2004). The citation (17) of Kettles et al., 1997 refers to a paper on epidemiology in humans and appears to be cited out of context. Discussion of studies on *Caiman latirostris* fails to mention that no effects on sex ratio were observed in eggs treated topically with 15 µg atrazine/egg dissolved in 50 µL of ethanol (Beldomenico et al., 2007). The relevance of effects of treatment with 200 µg atrazine/kg egg on the diameter of seminiferous tubules (Rey et al., 2009) in the same species is uncertain as there were no responses in proliferative activity, apoptosis, cellular turnover in the seminiferous tubules, or on concentrations of testosterone. Hayes et al. (2010) also do not cite the lack of response to exposures to atrazine on eggs of red-eared slider turtle (*Psuedemys elegans*) and *A. mississippiensis* to nominal aqueous exposures of as great as 500 µg atrazine/L used to drench the eggs (Gross 1999a, b) or the similar observations of lack of effect on morphology of the thyroid gland or number of testicular ovarian follicles in snapping turtles (*Chelydra serpentina*) exposed *in ovo* to atrazine via treated soil (De Solla et al., 2006).

Birds: Effects of atrazine cited for birds (Matsushita, 2006) were observed at higher concentrations than are relevant to exposures via water. Hayes et al. (2010) do not include data from this publication showing that exposures of Rhode Island Red and Plymouth Rock chickens *in ovo* had no effect on activity of aromatase in the gonad, sex ratio, or hatchability. There were no effects on development of the testes but there was an increased frequency (20%) of retention of the right gonad, which was incompletely differentiated in the affected animals. The significance of these observations on reproductive fitness, if any, is unknown. Hayes et al. (2010) omit data from other high dose studies showing that atrazine, up to 1,000 mg/kg in the diet (approximately 150 mg/kg/d), exhibited “inconsistent and modest” effects on the reproductive system during sexual maturation of the male quail (Wilhelms et al., 2005. Wilhelms et al. (2006b) injected quail eggs with doses atrazine as high as 504 µg/kg and concluded that “no evidence is presented that atrazine induces feminization of the testis in male quail”. As

well, Hayes et al. (2010) do not report the results of studies that have shown that dietary atrazine exhibits limited reproductive toxicity in female quail during sexual maturation and only at concentrations above ecological relevance (Wilhelms et al., 2006a).

Mammals: Effects observed in mammals were at very high exposures and relevance (or lack of) to risks in humans have been addressed (USEPA 2000).

- The statements that “demasculinization and feminization of amphibians in agricultural areas where atrazine is used (32) and directly correlated with atrazine contamination in the wild (7, 9, 33, 34)” are either incorrect or based on a biased selection of data. Du Preez et al., 2005b (Reference (32) did not report any effects related to “demasculinization and feminization”. In fact, no effects were observed. References 7 and 9 are to the same data set reported in (Hayes et al., 2003) which did not show a correlation to exposures. In fact, the site with the greatest frequency of frogs with testicular ovarian follicles (TOFs) had the lowest exposures to atrazine. Reeder, et al. (1998) (Reference 33) is misrepresented. In fact, Reeder et al. (1998) report no significant intersex differences in cricket frogs although they report an association “approaching significance”. Further, they recognize that “atrazine contaminated sites may represent areas of great agricultural impact, potentially involving other chemical, physical, and biotic factors” Further, while misrepresenting Reeder et al. (1998) to support their hypothesis, Hayes et al., (2010) do not note that these same authors (Reeder et al., 2005) found that the greatest incidence of intersex in cricket frogs occurred before the introduction of atrazine in agriculture. Murphy et al., 2006 (Reference 34) did not demonstrate consistent and concentration-related effects of atrazine on frogs in the field.
- The authors cite some studies that have not reported any effects of atrazine on testicular morphology in *X. laevis* but then suggest that this is due to “using different experimental conditions and different populations of the same species”. The authors:
 - incorrectly interpret the results of Carr et al. (2003). Carr et al. (2003) defined intersex as gonads that could not be clearly identified as testis or ovary because of shared or undifferentiated traits in size, shape, and physical appearance. Subsequent histological evaluation of the gonads from the 25-µg/L group, as originally described in Carr et al. (2003) confirmed that gonads identified as intersex based upon their outward appearance showed no evidence of mixed ovarian and testicular tissue as corrected by Carr in Solomon et al. (2008) and (Wolf 2007).
 - do not mention a lack of observed effects in other studies carried out under the similar experimental procedures as used by Hayes (Oka et al., 2008).

- do not mention that the frequency of induction of TOFs above background was not increased in any of the populations of *X. laevis* that have been examined (Du Preez et al., 2009; Kloas et al., 2009).

- The authors state that “a few studies suggest that atrazine has no effect on amphibians under certain laboratory conditions” (Kloas et al., 2009). This research which Hayes et al. (2010) describe as just a “suggestion” of no effects was in fact the only research conducted under full GLP conditions of data documentation, transparency and inspection by US EPA and German regulatory authorities (Grim and Steeger, 2008). This is a robust study, conducted in duplicate laboratories, designed in part by the EPA and the Scientific Advisory Panel to specifically address the hypothesis purported by Hayes that atrazine alters the sexual development of amphibians. Based on these results, the EPA concluded “that atrazine does not adversely affect amphibian gonadal development” (USEPA 2007a). A panel of independent expert scientists convened by the USEPA agreed that “reproductive fitness (sex ratio, intersex condition) were unaffected” in *X. laevis* at levels up to 100 µg/L and “there is currently no available proof for the hypothesis regarding the purported action of atrazine on the induction of aromatase” (USEPA 2007b). Hayes’ research has never approached this level of scientific transparency or rigor.

- Of the studies that do not report responses similar to the effects reported by Hayes, Hayes et al. (2010) only cite one (Kloas et al. 2009). Laboratory and field studies that show no adverse effects of atrazine on frogs have not been referenced (Jooste et al., 2005b, a, Du Preez et al., 2005b, Du Preez et al., 2008, Du Preez et al., 2009, Smith et al., 2005, Coady et al., 2004, Coady et al., 2005, Hecker et al., 2004, Hecker et al., 2005b, Hecker et al., 2005a, Murphy et al., 2004a, b, Oka et al., 2008, McDaniel et al., 2008). Instead, the focus in Hayes et al. (2010) is on seriously flawed studies such as those of Tavera-Mendoza (discussed above) and an over-interpretation of data on *R. pipiens* metamorphs exposed to 10 µg atrazine/L in which Orton et al. (2006) reported no difference in the total number of spermatogenic cells. Orton et al. (2006) did report an increase in the percentage of testicular cells in the latter stages of spermatogenesis relative to controls (38% vs. 20% in the controls) but the biological significance of this effect is unclear since there were no other effects of atrazine on testicular development and only one atrazine concentration was tested. The citation (32) to Du Preez et al. (2005b) is misrepresented as they did not report any effects in atrazine-exposed wild-type frogs.

- The authors suggest that other studies examining long-term transgenerational effects of atrazine (Du Preez et al., 2008) are flawed by the use of human chorionic gonadotropin,

(hCG), to stimulate mating. The authors also suggest that this was “effectively providing hormone replacement therapy” and “reversed the effects of atrazine.” The objective of the transgenerational study (Du Preez et al., 2008) was to assess reproductive success in the F0 frogs and transgenerational effects in the F1 metamorphs. Because *X. laevis* in the wild breed in response to complex environmental cues (Balinsky 1969), hCG is commonly used to stimulate mating under laboratory conditions and was used by Hayes et al. (2010) in females in their Mate Choice tests. There is no evidence that injection of hCG into frogs will reverse any physical effects, much less in the short time (hours) between treatment and mating.

- The authors claim that “hermaphroditism observed at metamorphosis in animals exposed to atrazine (6, 10) can ultimately result in complete feminization.” But they have not demonstrated that these same conditions of “hermaphroditism” existed at metamorphosis in the “males” used in this study. Data from Carr et al. (2003) is incorrectly cited as demonstrating hermaphroditism and the reference to Hayes et al. (2002a) reported on “feminization” of the laryngeal dilator muscle, which is not reported in Hayes et al., (2010). There is no biologically plausible mechanism to support these findings.
- As support for their argument of feminization, the authors quote the results of other studies (Suzawa and Ingraham, 2008, Oka et al., 2008, Langlois et al., 2009) without a critical evaluation. The extrapolation of these data to the statement “that sex-reversal by atrazine (complete feminization of genetic males) is not a species-specific effect but rather one that occurs across nonamniote vertebrate classes” is not scientifically valid.
 - The reported effects in the zebrafish (Suzawa and Ingraham, 2008) are highly questionable because of inconsistency in the data related to numbers of animals used in the study (See above).
 - In the studies by Oka et al. (2008) and Langlois et al. (2009) the apparent feminization of the frogs was the result of a male-dominated sex ratio in the controls. The percentage of wild-type males in the controls in the Oka et al. (2008) study was 60.8% which was greater than the expected nominal 50% and likely resulted in the significant differences observed. In fact Oka et al. (2008) concluded 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.” In Langlois et al. (2009), the reported effect on sex ratio was not due to an unusual ratio in the treated animals (42% male) but rather a skewed ratio in the control animals (62% male). Others authors, report sex ratios in control animals closer to the expected 50% males:

50% female ratio in the same species (*R. pipiens*). Chen et al., (2009) reported 54.0%±2.8 males and Hogan et al. (2008) reported the percentage of males ranged from 45 to 50%, based on research conducted in the same laboratory as Langlois et al. (2009). This suggests that the animals used in the study by Langlois et al. (2009) were unusual in some way or more likely that there was, by chance, biased selection of experimental animals.

- Hayes et al, (2010) do not discuss the many other studies that do not support their hypothesis since these have not reported any change or a concentration-related change in sex ratio as a result of exposure to atrazine (Jooste et al., 2005b, a, Du Preez et al., 2005b, Du Preez et al., 2008, Du Preez et al., 2009, Smith et al., 2005, Coady et al., 2004, Coady et al., 2005, Hecker et al., 2004, Hecker et al., 2005b, Hecker et al., 2005a, Murphy et al., 2004a, b). Two studies additionally reported no effects of environmentally relevant atrazine concentrations on three different native North American species (LaFiandra *et al.*, 2008; Storrs and Semlitsch, 2008). These studies are significant since (a) both used static- or static-renewal exposure systems and (b) both tested the potential effects of atrazine on amphibian species with significantly different life histories and developmental rates than *X. laevis*.”
- The comment that “there was a decline in all testosterone dependent morphologies examined here, including demasculinized/feminized laryngeal morphology and decreased breeding gland size” fails to acknowledge that for the larynx and breeding gland many measures of growth and morphology were assessed, yet 5 out of 6 measurements were statistically non-significant. Additionally, the numerous studies (cited above) that fail to demonstrate any effects on testosterone-dependent responses are neglected.
- The reported findings of Tavera-Mendoza et al. (2002a, 2002b) that the “previously reported decline in germ cells and nursing cells after only 48 h exposure to atrazine” are problematic. These results are inconsistent with the thesis upon which they are based (Tavera-Mendoza 2001) and therefore not repeatable in the same laboratory. Incomplete descriptions of the methods and inconsistencies in the data by Tavera-Mendoza et al. (2002a, 2002b) and the thesis (Tavera-Mendoza 2001) are not supportive of this reported finding.
- The authors state that “the demasculinized larynges suggest that the smaller laryngeal size observed at metamorphosis in previous studies (10, 41) results in persistent effects through sexual maturity.” The observations in the paper (10) (Hayes et al., 2002a) have not been replicated in other studies such as those of Carr et al. (2003). The reference (41) is to Hayes (2005), a polemic in which no new experimental data were reported. This

purported response in laryngeal size has not been observed in other studies in the field (Smith et al., 2005).

- Moore and Waring (1998) are cited as being consistent with the results of the present study, however.. The conclusions in Moore and Waring (1998) were compromised by incorrect statistical analysis (although it did show an effect of atrazine exposure on the responses of the olfactory epithelium). A second experiment (Moore and Lower, 2001) with exposures of salmon to concentrations of atrazine similar to those used by Hayes et al. (2010) did not cause changes in the concentrations of testosterone, 11-ketotestosterone or 17,20- β -dihydroxy-4-pregnen-3-one, compared to control fish that were also exposed to female priming pheromone. Data from a USEPA (2005) study in which this response was not observed is not referenced. In this 21-d reproduction bioassay *Pimephales promelas* were exposed to atrazine at measured concentrations of 25 and 224 $\mu\text{g/L}$ and no treatment-related effects were reported on estradiol or testosterone in females or testosterone and 11-ketotestosterone in males (USEPA 2005).
- Although Hayes et al. (2010) claim that exposures of eggs to 200 μg atrazine/kg reduced “sperm content in a reptile (caiman, *Caiman latirostris*), producing a morphology nearly identical to what we report here”, this is not specifically stated in the paper referenced (Rey et al., 2009). These authors made no evaluation of sperm content. Also inconsistent is the lack of response in several other endpoints reported in Rey et al. (2009). The only responses to treatment of eggs of the caiman with atrazine were an increase in the perimeter of seminiferous tubules in hatchlings and a reduction in proportion of the perimeter of the tubules occupied by desmin-positive cells (likely a result of the increased perimeter) (Rey et al., 2009). There were no responses in proliferative activity, apoptosis, cellular turnover in the seminiferous tubules, or on concentrations of testosterone.
- Hayes et al. (2010) cite studies in rodents and other animals as supportive of their observations in frogs. The authors acknowledge that exposures were greater in these studies but do not mention that these doses 100 – 200 mg/Kg bw via oral administration, are much greater than those to which frogs or other aquatic organisms (or mammals) are likely to be exposed. For example, based on a Biological Concentration Factor (BCF) of 1.5 for atrazine in *X. laevis* (Edginton and Rouleau, 2005), doses in rodents are equivalent to exposures of 66,000 -133,000 $\mu\text{g/L}$ in water, well above the maximum water solubility of atrazine (33,000 $\mu\text{g/L}$) and several orders of magnitude greater than typical environmental concentrations, as well as those tested by Hayes et al. (2010).
- Hayes et al. (2010) persist in stating that the responses in frogs are mediated through the induction of aromatase yet they offer no evidence of this in the males from their own

study and they do not mention the large number of studies that have concluded that aromatase induction does not occur in frogs or other aquatic organisms. Exposures to atrazine did not result in the induction of aromatase in frogs (Hecker et al., 2004, Murphy et al., 2006, Oka et al., 2008, Hecker et al., 2005a). Aromatase was not induced in one study on zebrafish exposed to concentrations as great as 2,150 µg/L (Kazeto et al., 2004). The only report of induction of aromatase in fish (Suzawa and Ingraham, 2008) is confounded by the lack of replication, pooling of samples for analysis, and lack of a negative control such as was used in Kazeto et al. (2004). A panel of independent expert scientists convened by the US EPA in 2007 agreed that “there is currently no available proof for the hypothesis regarding the purported action of atrazine on the induction of aromatase” (USEPA 2007b).

- Hayes et al. (2010) cites studies (Du Preez et al., 2005a, Du Preez et al., 2005b) reporting atrazine exposures of frogs in the wild similar to the dose tested in Hayes et al. (2010) thereby suggesting “the impacts of atrazine on amphibians and on wildlife in general are potentially devastating. However, not reported in Hayes et al. (2010) is that these concentrations were observed in sites with robust populations of frogs. Du Preez et al. (2005a and 2005b) reported robust populations of frogs in sites in South Africa where *X. laevis* have been exposed to low levels of atrazine for decades. No effects on sex ratio or primary and secondary sexual characteristics were reported (Du Preez et al., 2005b, Smith et al., 2005).
- The statement that “semi terrestrial frog species take up significant amounts of atrazine (51)” is not supported by the cited reference. The paper by Storrs Mendez et al. (2009) utilized ¹⁴C-labelled atrazine to study uptake in terrestrial stages of *Buffo Americans*. Animals were exposed to concentrations of atrazine equivalent to 460 µg/L. This concentration is not environmentally relevant and insufficient details are given in the paper to determine the amount taken up on the basis of the body mass or organ mass. The actual body dose is not given and this study provides no information useful for assessing potential risk of atrazine to amphibians. However, the distribution of the atrazine in the organs, with large amounts in the gall bladder and intestine, suggest rapid metabolism and/or excretion as was observed in *X. laevis* (Edginton and Rouleau, 2005).
- Results in this publication are inconsistent with other publications by Hayes which consistently report findings of multiple gonads and hermaphroditic frogs with multiple testes and ovaries.
 - Hayes has previously reported that concentrations of atrazine as low as 0.1 µg/L caused partial feminization (mixed sex hermaphrodites) in frogs when they were exposed during the sensitive developmental window, during metamorphosis. Hayes et al. (2002) states that “At all doses tested (except 0.01 ppb), atrazine

produced gonadal abnormalities. Up to 20% of the animals (16-20%) had multiple gonads (up to 6 in a single animal) or were hermaphrodites (with multiple testes and ovaries). This percentage was subsequently revised to 32-40% of the exposed males (Hayes et al., 2006).

- In a study with atrazine-exposed leopard frogs, Hayes et al. (2003) reported a finding “of 29% of the 0.1 µg/L-treated animals and 8% of the animals treated with 25 µg/L displaying varying degrees of sex reversal”, clearly showing no dose response.
- But in this study (Hayes et al., 2010) the authors report that 10% of the adult ZZ males were completely feminized, and the remaining 90% exhibited neither testicular ovarian follicles (TOFs) nor hermaphroditism as repeatedly reported for juvenile (NF Stage 66) animals in his previous publications (Hayes et al., 2002; Hayes et al., 2003, Hayes et al., 2006). There is no indication by the authors that this conflicts with the original findings from Hayes et al. (2002, 2003, and 2006); therefore the presumption is that the males in this study also exhibited mixed sex during early development through sexual differentiation. To be consistent with his previous publications of 32-40% hermaphrodites and multiple gonads at concentrations as low as 0.1 ppb, one might expect even higher percentages of these male frogs exposed to 2.5 µg/L to exhibit like gonadal malformations. Such is not the case.
- Rather the authors now suggest that, after long-term exposure to 2.5 µg/L atrazine through sexual development, some juvenile mixed-sex animals apparently become fully feminized adults while the remainders exhibit no gonadal malformations. The reported findings therefore suggest that longer-term exposure of males which were either unaffected at metamorphosis, or of hermaphroditic condition at metamorphosis, such as reported in Hayes’ previous work, subsequently matured as partially feminized males, but lacking the hermaphroditic condition. The fact that only 10% of the frogs in the current study were feminized also suggests that some frogs of hermaphroditic condition at metamorphosis apparently reverted back to males with normal testes.
- This purported result is inconsistent with the fact that after passing through the sensitive developmental window which begins at NF Stage 45-47, the gonads become “fixed” as males, females or, when exposed to estradiol at low concentrations “mixed sex” hermaphrodites. Villalpando and Merchant-Larios (1990) established that exposure to even high concentrations of estradiol benzoate after developmental Stage 55-56 but prior to complete metamorphosis did not affect the development of the testes of male *X. laevis*. Gyllenhammar et al., (2009) exposed *Xenopus tropicalis* to a range of ethynylestradiol (EE2) concentrations beginning 4-5 days after hatching until approximately 2 months post-metamorphosis. Animals were examined at sexual maturity (approximately

8 months post-metamorphosis) and in addition to the expected increase in female phenotype animals, intersex and testicular oocytes or testicular ovarian follicles (TOFs) were observed. The persistence of effects produced by EE2 during metamorphosis has been further demonstrated. Pettersson et al., (2006) exposed *X. tropicalis* to EE2 beginning 4-5 days after hatching through metamorphosis and examined the reproductive organs 1 month post-metamorphosis (juveniles) and at 9 months (sexual maturity). The authors conclude that transient early life-stage exposure to EE2 can induce feminizing effects that persist into adulthood. These studies do not indicate the capability of post-metamorphosis, phenotypic plasticity as is suggested by Hayes et al., (2010).

- Hayes et al. (2010) is inconsistent with other publications by Hayes relative to measured testosterone levels. The authors report reduced concentrations of plasma testosterone in frogs exposed to atrazine. The testosterone levels reported in both control and atrazine treated frogs are inconsistent with concentrations previously reported by Hayes et al. (2002a) in PNAS. Sexually mature *X. laevis* control males were reported to have mean plasma testosterone levels of 4 ng/mL while males treated with 25 µg atrazine/L had mean levels of approximately 0.5 ng/ml (Hayes et al., 2002a, Fig 4). In contrast, Hayes et al. (2010) reports concentrations in plasma of control males ranging from 0.0 to 40 ng/mL and in atrazine-treated males from 0.0 to 25 ng/mL. While variability in this metric is expected, this variability is large and questions the utility of drawing conclusions from this biomarker. The authors did not analyze for plasma estradiol which should have been elevated to confirm their results and conclusions regarding aromatase. Additionally, the authors did not refer to other studies in which there are no effects from atrazine on concentrations of testosterone and estradiol observed in the laboratory (Coady et al., 2005, Hecker et al., 2005a) or the field (Hecker et al., 2004).
- The responses in the larynx of the ZZ males are not consistent with previous studies for wild-type *X. laevis* conducted in the same laboratory. In this paper and the SI, the authors state that the shape of the larynx was altered by the exposure to atrazine but that there were no effects on “laryngeal weight (after fixation), laryngeal weight corrected for body weight, laryngeal length, laryngeal length corrected for body size, and maximum cross-sectional area (transverse) of the *dilater laryngis* muscle” (actually consistent with no effects observed by (Carr et al. (2003) and Smith et al. (2005)). A change in laryngeal shape but not in 5 other laryngeal measurements is contrary to previous results. Hayes et al. (2002a) reported significant decreases in the cross-sectional area of the *dilater laryngis* muscle.

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