Data Evaluation Report on Forms and Prevalence of Intersexuality and Effects of Environmental Contaminants on Sexuality in Cricket Frogs (*Acris crepitans*)

EPA MRID Number: None

Data Requirement:

EPA DP Barcode None

EPA MRID Not Assigned
EPA Guideline Open Literature

Test material: **Purity:** not reported

Common name Atrazine
chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
synonyms
EPA PC Code: 80803

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CITATION: Reeder, A. L., G. L. Foley, D. K. Nichols, L. G. Hansen, B. Wikoff, S. Faeh, J. Eisold, M. B. Wheeler, R. Warner, J. E. Murphy, and V. R. Beasley 1998. Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). Environmental Health Perspectives 106 (5):

EXECUTIVE SUMMARY:

In an effort to assess the prevalence of gonadal intersexuality in adult and juvenile cricket frogs *(Acris crepitans*) and to determine whether sexuality is altered in response to exposure to environmental contaminants, cricket frogs were collected over a three year period (1993 - 1995) in various locations throughout the state of Illinois. Additionally, water/sediment samples were collected at sampling sites in 1994 and 1995 to determine whether the prevalence of intersex could be related to chemical residues. In a separate study, cricket frogs were also collected at a site known to be contaminated with PCBs and PCDFs and the prevalence of intersex was determined relative to control sites.

Of the 55 adult and juvenile male and female frogs collected in 1993, 2 (3.6%) had both an ovary and testis. In the testis of one, spermatogenesis was normal; in the other, an immature ovary was present as well as a testis with no active spermatogenesis. Of the 243 frogs examined in 1994, 6 (2.5%) contained both ovary and testis; five of the affected animals had areas of normal spermatogenesis in the testis interspersed with oocytes. One animal had a mature ovary and mature testis with normal spermatogenesis. Of the 43 frogs examined in 1995, only one (2.3%) had an ovotestis. Across all three sampling years the prevalence of intersex was 2.8%. In specimens with an ovary on one side and an testis on the other, ovarian size ranged form well-developed mature female to extremely small with a few oocytes present.

Of the five sites where intersex was found, four had detectable atrazine. Of the four sites where no intersex was observed, only one contained detectable levels of atrazine. According to the authors the relationship between detection of atrazine and prevalence of intersex "approached significance" (P = 0.07). At one site treated with copper sulfate in 1994, 1 frog of 33 collected had an ovotestis. In 1995, no relationship between the detection of atrazine and the prevalence of intersex. No intersex was identified in frogs collected from a pond treated with endothall in 1995. Lead residues measured in 1994 and 1995 were not associated with the prevalence of intersex.

Of the frogs collected from PCB and control sites, only 1 frog with an ovotestis was identified from the control. Sex ratios differed significantly (probability not given) between contaminated and control sites. In 13 juveniles from control and 13 from contaminated sites, gonadal tissue was immature and could not be identified for histological preparation. According to the authors, the association between sex ratios of PCB/PCDF contaminated and control groups revealed a significant difference (p = 0.0007).

While a wide range of chemical residue analyses were conducted, no data are provided on the results of these analyses. It is not clear why the study focuses primarily on atrazine; however, the authors suggest that there may be a trend between atrazine residues and the proportion of animals exhibiting intersex. The only statistically significant relationship though was between sex ratios in PCB/PCDF contaminated sites relative to controls; however, the sample size for making this determination was low (n=4).

In this paper, Reeder *et al.* (1998) discussed the range of chemical residues in the field collection sites and how these chemicals, combined with environmental conditions, could impact gonadal development. These factors contributed to the limited utility of this study because the investigation did not demonstrate a significant effect of chemical residues on the prevalence of intersex in cricket frogs. This study underscored the need to have focused study designs with sufficient power in terms of sample size to discriminate effects if they exist. Also, the report acknowledged that little is known about natural intersex rates in cricket frogs. Without a better

understanding of the biology of the cricket frog and the toxicological phenomenon being examined, it is difficult to interpret the significance of the reported observations.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study
COMPLIANCE: Not conducted under full Good Laboratory Practices

A. MATERIALS:

1. Test Material Atrazine

Description:

Lot No./Batch No. : Not reported

Purity:
Stability of Compound
Under Test Conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: Cricket frogs (Acris crepitans)
.
Age at test initiation:
Weight at study initiation: (mean and range) not reported
Length at study initiation: (mean and range) not reported

Source: Field-collected along pond banks in sites distributed across Illinois

B. STUDY DESIGN:

Objective: to assess the prevalence of gonadal intersexuality in adult and juvenile cricket frogs *(Acris crepitans*) and to determine whether sexuality is altered in response to exposure to environmental contaminants

1. Experimental Conditions

a) Range-finding Study: In 1993, a pilot study focused on pond site identification and preliminary assessment of histologic lesions in Illinois cricket frogs, 20 sites selected.

b) Definitive Study

In 1994 and 1995 frogs were collected from specific sites based on suitable habitat and presence of cricket frogs; ponds were of comparable size and were distributed across the state of Illinois.

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: conditions: (same as test or not) Feeding: Health: (any mortality observed)	NA
Duration of the test	NA
Test condition static/flow through Type of dilution system- for flow through method. Renewal rate for static renewal	NA
Aeration, if any	NA
Test vessel Material: (glass/stainless steel) Size: Fill volume:	NA
Source of dilution water Quality:	NA

Parameter	Details
Water parameters: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature Salinity Intervals of water quality measurement	NA
Number of replicates/groups: negative control: 0.004% ethanol treated ones:	NA
Number of organisms per replicate /groups:	In 1993, gonads of 50 intact cricket frogs from 18 sites. In 1994, gonads of 242 frogs examined from 8 sites (24 - 39 frogs from the various sites). In 1995, gonads of 40 frogs examined from 7 sites (4 - 7 frogs per site).
Biomass loading rate	NA
Test concentrations: nominal:	NA
Solvent (type, percentage, if used)	NA
Lighting	NA
Feeding	NA

Parameter	Details
Recovery of chemical Level of Quantitation Level of Detection	NA
Positive control {if used, indicate the chemical and concentrations}	NA
Other parameters, if any	NA

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	prevalence of gonadal abnormalities in terms of testicular oocytes.
Observation intervals	NA
Were raw data included?	No
Other observations, if any	

Study A

In 1994, eight sites with similar size studied (5 in south, 2 in central and 1 in northeast Illinois). In 1995, seven of the ponds studied in 1994 and one pond in the agricultural area of east-central Illinois, studied.

In 1993, gonads of 50 intact cricket frogs from 18 sites in east-central and southern Illinois examined for gross morphology. In 1994, gonads of 242 frogs examined from 8 sites (24 - 39 frogs from the various sites). In 1995, gonads of 40 frogs examined from 7 sites (4 - 7 frogs per site).

Animals from 1993 subject to whole body histologic sections while samples from 1994 and 1995 were subject to gonad histology.

Composite water and sediment samples collected in acetone-rinsed containers from multiple locations in each pond studied in 1994 and 1995. Samples collected at the end of May and again in late

July/early August in 1994 and at the end of May in 1995. Additional water samples were collected at sites 11 and 12 in June 1998 following an aquatic plant kill. Water analyzed (State of Illinois Animal Disease Laboratory, Centralia, IL) for herbicides, fungicides, insecticides, PCBs and metals (**Table 1; source: Reeder *et al.* 1998**).

Study B

In mid-July 1995, 16 juvenile cricket frogs collected from three sites in Crab Orchard National Wildlife Refuge in southern Illinois where point contamination with PCBs, PCDFs, and PCDDs had previously occurred. On the same sampling days, 16 juvenile frogs also collected from each of three control sites located outside the area of PCB and PCDF contamination but within a 16-km radius of the Crab Orchard site.

Table 1. Compounds analyzed in water and sediment samples (water detection limits in parentheses, µg/l)

Herbicides	Insecticides
Alachlor (5)	Carbamates
Ametryn (1)	Aldicarb (10)
Atrazine (0.5)	Aminocarb (5)
Barban (5)	Bendiocarb (5)
Bifenox (5)	Carbaryl (5)
Bromacil (1)	Carbofuran (5)
Butachlor (5)	Lannate (5)
Butylate (5)	Methiocarb (5)
Chloropropham (5)	Oxamyl (5)
Chlorothal (5)	Organochlorine
Cyanazine (0.5)	Aldrin (0.5)
Diclofop (5)	Chlordane (0.5)
Dinitramine (5)	DDD (0.5)
Dipropetryn (5)	DDE (0.5)
Diuron (5)	DDT (0.5)
EPTC (5)	Dieldrin (0.5)
Fluchloralin (5)	Endosulfan (0.5)
Hexazinone (5)	Endrin (0.5)
Linuron (5)	Heptachlor (0.5)
Metolachlor (5)	Heptachlor epoxide (0.5)
Metribuzin (5)	Lindane and isomers (0.5)
Monuron (5)	Methoxychlor (0.5)
Napropamide (5)	Mirex (0.5)
Napralam (5)	PCBs (0.5)
Oryzalin (5)	Organophosphorous
Pebulate (5)	Chlorpyrifos (1)
Pendimethalin (5)	Diazinon (1)
Profluralin (5)	Dimethoate (1)
Prometon (5)	Disulfoton (1)
Prometryn (5)	Ethoprop (1)
Propanil (5)	Ethyl parathion (1)
Propazine (5)	Fenchlorphos (1)
Propham (5)	Fenthion (1)
Simazine (1)	Fonofos (1)
Terbuthylazine (5)	Isofenphos (1)
Terbutryn (5)	Malathion (1)
Trifluralin (5)	Methidathion (1)
	Methyl parathion (1)
	Mevinphos (1)
	Phorate (1)
	Terbufos (1)
	Trichlorfon (1)
	Fungicides
	Benomyl (5)
	Hexachlorobenzene (0.5)
	Thiobencarb (10)
	Metals
	Lead (5, 0.1 mg/kg*)
	Mercury (0.1 µg/kg*)

*Sediment detection limits.

Fisher's Exact Test used to compare prevalence of intersex gonads and detection of atrazine at those sites. Fisher's Exact test also used to compare sex ratios among control and PCB/PCDF sites using SigmaStat software.

II. RESULTS and DISCUSSION:

Study A

Although cricket frog testicular tissue is typically heavily pigmented, about 20% of males had testes with reduced or absent pigmentation particularly on the right side; however, normal spermatogenesis was observed in both pigmented and nonpigmented testes. Mature females contained large egg mass with bipolar eggs (tan/black coloring).

Of the 55 adult and juvenile male and female frogs collected in 1993, 2 (3.6%) had both an ovary and testis. In the testis of one, spermatogenesis was normal; in the other, an immature ovary was present as well as a testis with no active spermatogenesis. Of the 243 frogs examined in 1994, 6 (2.5%) contained both ovary and testis; five of the affected animals had areas of normal spermatogenesis in the testis interspersed with oocytes. One animal had a mature ovary and mature

testis with normal spermatogenesis. Of the 43 frogs examined in 1995, only one (2.3%) had an ovotestis. Across all three sampling years, the prevalence of intersex was 2.8% (report claims 2.6%). In specimens with an ovary on one side and an testis on the other, ovarian size ranged form well-developed mature female to extremely small with a few oocytes present.

Of the five sites where intersex was found, four had detectable atrazine. Of the four sites where no intersex was observed, only one contained detectable levels of atrazine. According to the authors the relationship between detection of atrazine and prevalence of intersex "approached significance" (P = 0.07). At one site treated with copper sulfate in 1994, 1 frog of 33 collected had an ovotestis. In 1995, no relationship between the detection of atrazine and the prevalence of intersex. No intersex was identified in frogs collected from a pond treated with endothall in 1995. Concentrations of lead from both years not associated with intersex either.

Study B

Of the frogs collected from PCB and control sites, only 1 frog with an ovotestis was identified from the control. Sex ratios differed significantly (probability not given) between contaminated and control sites. In 13 juveniles from control and 13 from contaminated sites, gonadal tissue was immature and could not be identified for histological preparation. According to the authors, the association between sex ratios of PCB/PCDF contaminated and control groups revealed a significant difference (p = 0.0007).

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F. REVIEWER'S COMMENTS:

It is not clear what was driving the sampling design used in this study. Sample size varied considerably between years: 1993 (55 frogs), 1994 (243 frogs) and 1995 (43 frogs).

There are several discrepancies between the tables and the text. In discussing the prevalence of intersex relative to atrazine, the authors refer to 5 sites with residues and 4 sites without residues in the 1994 sampling; however, according to the methods section only 8 sites (not 9) were sampled in 1994.

In study A, a wide range of chemicals were analyzed in both water and sediment at each of the collection sites in 1994 and 1995; however, the results section does not discuss the outcome of these analyses but rather focuses primarily on atrazine thus implying that it was either the only chemical were residues were detected or that it was the only chemical residues where the researchers could

identify a significant relationship. Since no data are provided on residue levels, it isn't possible for the Agency to verify whether a correlation existed between atrazine residues and the prevalence of intersex; however, the results section suggests that if a relationship did exist in 1994, it was transient since no relationship existed in 1995. Further, it isn't clear whether the lack of a "relationship" between atrazine residues in 1995 and atrazine residues was a result of only one intersex animal being found in spite of atrazine residues or whether there were no detectable atrazine residues. The article implies that the authors attempted to correlate atrazine residues only to cases where intersex was determined. If this is the case then the analysis is somewhat biased since zero prevalence would not be captured. Atrazine residues at each sites should have been compared to the rate of intersex at each site; however, it doesn't appear that the study was designed to anticipate such an analysis. The utility of calculating an overall prevalence of abnormalities seems unsound, as it unnecessarily combines data from sites identified as contaminated or control.

In study B, the methods section suggests that they collected 16 frogs from control and from contaminated sites. The results section states that the gonads of 13 frogs from each location were too immature to determine sex. This implies that over 80% of the animals were too immature to determine sex. Therefore, the effective sample size for determining sex ratio differences was low with three animals in each group.

Presentation of the analytical chemistry data is not clear...are blank entries indicative of no analyses or no measurable quantities? The authors note the variety of chemical residues measured in the field studies and how varying levels of response to the wide range of chemicals might influence the frog's sensitivity to the chemicals. Classifying a site categorically as atrazine contaminated based on the sampling plan is misleading. The real question is: what were the atrazine concentrations during the developmental stage of interest? Given this lack of information, it is difficult to accept the correlational association of gonadal abnormalities and atrazine presence, as it has no basis in the biology nor toxicology. Additionally the authors note how environmental factors such as temperature may impact gonadal development. Given the number of potentially confounding effects, it is difficult to place much weight on the statistical relationships developed in what amounts to a reconnaissance survey. These factors contribute to the limited utility of this study since the study does not demonstrate a significant affect of chemical residues on the prevalence of intersex in cricket frogs. The study underscores to need to have focused study designs with sufficient power in terms of sample size to discriminate effects should they exist.

The report admits in the introduction that virtually nothing is known about intersex conditions in cricket frogs. Perhaps cricket frogs exhibit ovotestes normally at some low level and the presence and absence of this condition at any one site is a function of small sample sizes. In general, this study is another one that puts the "cart ahead of the horse." That is, they conducted a field study, replete with complications and uncertainties without having a firm understanding of either the biology of the organism that they were studying or the toxicological phenomenon that they attempted to study. It clearly does not contribute to the understanding of whether or not atrazine poses any reproductive risk to anurans.