In Ovo Exposure to Atrazine on Circulating Reproductive Hormones and Gonadal Histology in Japanese Quail

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Summary and Implications

The triazine herbicide, atrazine, has come under scrutiny for its reported feminizing effects in amphibians. To date, there is little information concerning the effects of atrazine on reproduction in avian species. The current study examines the putative reproductive toxicity of atrazine after exposure *in ovo*. Atrazine at 504, 246 and 123 µg/kg was administered to Japanese quail eggs prior to incubation. The eggs were hatched and the birds raised to 14-days of age. Indices of hatchability, sex ratios and growth were determined. Furthermore, circulating concentrations of reproductive hormones (estradiol, progesterone and testosterone) and gonadal histology were examined. Atrazine at 504 µg/kg decreased 14-day hatchling weight 13.1% versus control. However, no detrimental effects on hatchability or sex ratios were observed. In female birds, atrazine at 504 µg/kg reduced ovarian weights and circulating concentrations of progesterone 48.3 and 73.3%, respectively, versus control. However, concentrations of estradiol and testosterone did not differ from control. In male quail, at all doses tested, atrazine reduced circulating concentrations of testosterone as much as 80.2% versus control. However, no effects on gonadal weights or circulating concentrations of estradiol or progesterone were observed. Moreover, no incidences of left ovotestis formation were observed. In contrast, 10 ng/kg ethinylestradiol (a positive control) induced the formation of a left ovotestis in 4 of 8 birds analyzed. The current results may suggest that exposure to atrazine *in ovo* at concentrations above ecological relevance exerts modest effects on the reproductive system of the young male and female quail. However, no evidence is presented that atrazine induces feminization of the testis in the male quail.

Introduction

Atrazine is a chloro-s-triazine herbicide that inhibits photosynthesis in plants. It is widely used in the United States, with up to 36,000 metric tons of field application/y (United States Environmental Protection Agency [USEPA] 2004). It has been established that atrazine exhibits low acute toxicity in terrestrial animals.

Although little acute toxicity is observed, atrazine exhibits reproductive toxicity in mammals. For example, in male rats, atrazine at 100 mg/kg reduces serum and testicular concentrations of testosterone and serum concentrations of luteinizing hormone (LH). Atrazine at 200 mg/kg increases serum concentrations of estradiol and estrone in rats undergoing sexual maturation, but inhibits steroidogenic enzymes, including 5α-reductase and 17β-hydroxysteroid dehydrogenase. In female rats, atrazine at 100 mg/kg inhibits LH release and induces a persistent state of estrus. Furthermore, atrazine suppresses the release of prolactin and inhibits mammary development. Recently, it has been reported that low concentrations of atrazine (100 ng/L) may exert feminizing effects in amphibians.

The egg is a potential route of exposure for toxicants in birds; it possesses high concentrations of lipid and thus may act as a sink for lipophilic compounds. Exposure to toxicants *in ovo* may be particularly important for precocial birds, such as the Japanese quail. In these birds, sexual differentiation occurs during embryonic development – a process highly susceptible to influence by exogenous hormonal signals. As such, recent studies have established the avian egg as a model for *in ovo* exposure to putative reproductive toxicants, some of which are classified as endocrine disruptors. In these studies, endpoints such as gonadal histology, circulating concentrations of reproductive hormones and reproductive behavior in adults have been assessed.

Based on the recent studies in amphibians and the fact that exposure to atrazine influences the hypothalamus-pituitary-gonadal axis in mammals, it may be hypothesized that atrazine exerts a negative effect on differentiation and development of the avian reproductive axis. Specifically, atrazine may exert a feminizing effect on the development of the reproductive system in the bird embryo The following study examines the putative reproductive toxicity of atrazine in the Japanese quail as exposed *in ovo*.

Materials and Methods

Animals and Procedure

Japanese quail (*Coturnix coturnix Japonica*) eggs were purchased from Lake Cumberland Game Birds, (Wayne, KY) and randomly assigned to six treatment groups (n = 27 per group). Quail eggs were injected using a Hamilton syringe with vehicle or test compound into the air cell and sealed with paraffin wax. They were then incubated at 37.5°C until hatch on days 17 and 18. Upon hatch, initial body weights were taken for each bird. On Day 19, hatch rates were determined and the remaining unhatched eggs were opened and assessed. All hatched birds were housed in battery cages on long photoperiods (16L:8D). The birds were provided feed and water *ad libitum*. At 2 weeks, the birds were reweighed,

decapitated at day 14 and blood samples taken into heparinized tubes. Plasma was separated by centrifugation (2500 x *g*, 10 min) and stored at –20°C until analysis. The ovary or testes were dissected out and weights recorded. The ovary or the left testis was then fixed in Bouin's solution for subsequent processing and histological examination.

Experimental Treatments

Japanese quail eggs were administered atrazine to determine its effects *in ovo*. Experimental groups were injected with atrazine in 50% propylene glycol at 500, 250 and 125 µg/kg egg (504, 246 and 123 µg/kg corrected for average egg weight within group). For comparison with known estrogenic effects, putative changes in reproductive endpoints due to atrazine were compared to eggs treated with 10 ng/kg 17α-ethinylestradiol (an estrogen positive control). This dose was based on those demonstrated to induce the formation of ovotestes in the developing quail.

Hormone Analysis

To identify atrazine-induced changes in circulating concentrations of reproductive hormones, plasma concentrations of estradiol, progesterone and testosterone were determined. All three steroid hormones were determined using ELISA systems. Circulating concentrations of estradiol were measured. The intra- and interassay coefficient of variation (CV) were 8.1 and 8.4%, respectively. Circulating concentrations of testosterone were determined. The intra- and interassay CV were 1.0 and 4.8%, respectively. The progesterone assay system was validated for use with quail plasma prior to use. Charcoal-stripped quail plasma was spiked with concentrations of progesterone equivalent to those supplied with the system. For the progesterone assays, the intra- and interassay coefficient of variation were 1.5 and 10.5%, respectively. In all assays, samples found to be below the lowest standard were assigned that concentration for statistical analysis (25, 150 and 62.5 pg/mL for estradiol, progesterone and testosterone, respectively).

Histological Analysis

To assess atrazine-induced changes in gonadal development, the left testis or ovary was processed for histological analysis. The tissues were fixed in Bouin's solution, washed in 70% ethanol 3 times and then stored in 70% ethanol at 4°C until further processing. The tissues were then dehydrated in 100% ethanol (2 changes), xylene (two changes) and then infiltrated with molten paraffin (three changes). The tissues were then embedded in paraffin blocks and sectioned at 7 µm.

Statistics

All statistics were performed using SAS version 9.0 (SAS Institute, Cary, NC). Bodyweights, gonadal weights and their associated somatic indices and circulating concentrations of estradiol, progesterone and testosterone were analyzed by one-way analysis of variance (ANOVA) using the GLM procedure. Where differences were found ($p < 0.05$), treatment means were compared with the vehicle control using Dunnett's *t*-test. Because of a lack of homogeneity of variance between groups, circulating concentrations of estradiol, progesterone, and testosterone were analyzed by nonparametric Kruskal-Wallis test. Where differences were found ($p < 0.05$), treatment means were compared with control using Dunnett's *t* test.

Results

Indices of General Toxicity

The effects of atrazine administered *in ovo* on indices of general toxicity are described in Table 1. Atrazine at 246 µg/kg egg had no influence on hatchability, sex ratios, day-1 body weights or hatchling survival to 14 days. Furthermore, atrazine exhibited no negative trends on feed intake or survival to 14 days. However, atrazine at 504 µg/kg egg decreased 14-day hatchling weight 13.1% versus control ($p < 0.05$).

Indices of Reproductive Toxicity

The effects of exposure to atrazine *in ovo* on indices of reproductive toxicity are presented in Table 2. In male birds, no effects of atrazine on testis weight or testis-somatic index were observed compared with controls (right testis not shown). Furthermore, atrazine did not influence the left-to-right testis weight ratio. Atrazine did not influence circulating concentrations of estradiol, testosterone, or progesterone (estradiol below the limit of detection in all cases) (Table 3). Similarly, ethinylestradiol did not influence testis weight or testis-somatic index. However, ethinylestradiol increased the left-to-right testis weight ratio 1.7-fold versus control ($p < 0.001$).

In female birds, atrazine at 504 µg/kg egg decreased ovary weight 48.3% versus controls ($p < 0.05$). However, no effects of atrazine were observed on the relative ovary weight versus control. Atrazine did not influence circulating concentrations of estradiol or testosterone. However, atrazine at 123 and 504 µg/kg egg decreased circulating concentrations of progesterone 73.3% and 75.2%, respectively, versus controls ($p < 0.05$) (Table 3). The positive control, ethinylestradiol, did not influence any of the indices examined.

Gonadal Histology

The effect of atrazine on gonadal development was examined through histological analysis. In male birds, control solvent or atrazine at all concentrations tested had no effect on general morphology or the presence of ovarian tissue within the testis. In contrast, 10 ng/kg ethinylestradiol induced the formation of ovarian tissue within the left testis in four of eight samples examined.

Discussion

The herbicide atrazine has come under scrutiny for its effects on reproduction and its endocrine disrupting potential in mammals. Atrazine administered *in ovo* did not influence weight or general morphology of the testis in Japanese quail. In contrast, the estrogen control, ethinylestradiol, induced the formation of a left ovotestis (oocytes present in the cortex of the testis) in four of eight samples examined. No oocytes were observed in the left testis of birds treated with atrazine *in ovo*. Atrazine did not increase the left-to-right testis weight ratio, an estrogenic response as observed in the positive control. This does not support the hypothesis that at the concentrations tested, *in ovo* exposure to atrazine exerts an estrogenic effect on the testes of the male bird. In contrast, in the female hatchling, atrazine decreased ovary weight, but not the relative ovary weight, versus controls. However, throughout all treatment and control groups, no overt differences were observed in the gross histology of the ovaries. This does not suggest that atrazine exerts a detrimental effect on the early development of the ovary. Alternatively, the effect may be attributed to the observed decrease in bodyweight.

Atrazine did not influence circulating concentrations of estradiol, progesterone, or testosterone in the 14-day hatchling male quail. Similarly, atrazine exerted little effect on estradiol and testosterone in the hatchling female quail. This change in circulating concentrations of reproductive steroids may suggest that atrazine influences reproductive steroidogenesis in the young female quail, an effect observed in other species.

It has been suggested that the feminizing effect of atrazine in male amphibians is caused by an increase in aromatase activity, increasing the conversion of androgen to estrogen. In these studies, circulating concentrations of testosterone were decreased, and the presence of ovarian tissue within the testes was observed. In the current study, atrazine did not influence circulating concentrations of testosterone or estradiol. Furthermore, ovarian tissue was not observed in the testes of any bird exposed to atrazine (up to 504 µg/kg egg). Moreover, atrazine administered in the diet to sexually developing male quail may increase circulating concentrations of testosterone. The current results do not support an increase in aromatase activity or an estrogenic response in the hatchling male bird. However, in the female hatchling, the change in circulating concentrations of progesterone may suggest an alternative effect not observed previously.

In summary, atrazine administered up to 504 µg/kg egg exhibited effects on the reproductive system of the 14-day female hatchling quail. However, the present results do not support an estrogenic effect of atrazine in the male hatchling exposed to high concentrations during *in ovo* embryonic development.

Table 1 Effect of *in ovo* exposure to atrazine on indices of general toxicity in the hatchling Japanese quail

	Indices of General Toxicity[a]								
Treatment	**Eggs**	**Hatched**	**%Hatched**	**Hatchling body weight(g)**	**Male**	**Female**	**14-day hatchling weight (g)**	**Daily feed intake (g/bird)**	**% Survival to 14 days**
Control	27	19	70.4	8.2 ± 0.20	13	5	61 ± 1.8	7.5	100
123 ng/g atrazine	27	19	70.4	8.4 ± 0.26	11	7	62 ± 1.8	8.1	95
246 ng/g atrazine	27	18	66.7	8.5 ± 0.20	12	6	59 ± 1.6	8.1	94
504 ng/g atrazine	27	20	74.1	8.5 ± 0.25	14	6	53 ± 1.5[b]	7.1	100
10 pg/g ethinylestradiol	27	19	70.4	9.0 ± 0.24	10	7	56 ± 2.8	6.6	90

[a] Results presented as mean ± SEM.
[b] Different from control at $p < 0.05$.

Table 2 Effect of *in ovo* exposure to atrazine on testis and ovary development in the 14-day male and female Japanese quail

	Indices of Male Development[a]					Indices of Female Development[a]			
Treatment	**Body weight(g)**	**Left testis weight(mg)**	**Relative testis weight (% body weight)**	**Left-to-right testis weightratio**	***n***	**Body weight(g)**	**Ovary weight(mg)**	**Relative ovary weight (% body weight)**	***n***
Control	60 ± 2.1	5.0 ± 0.53	0.008 ± 0.0008	0.9 ± 0.06	13	65 ± 3.4	29 ± 4.4	0.04 ± 0.006	4
123 ng/g atrazine	62 ± 2.7	5.4 ± 0.57	0.009 ± 0.0007	0.9 ± 0.02	10	63 ± 2.6	24 ± 2.4	0.04 ± 0.004	7
246 ng/g atrazine	60 ± 1.7	4.5 ± 0.50	0.008 ± 0.0008	0.9 ± 0.09	11	57 ± 3.3	21 ± 3.5	0.04 ± 0.004	6
504 ng/g atrazine	52 ± 2.6	3.7 ± 0.40	0.007 ± 0.0007	1.0 ± 0.08	12	54 ± 2.9	15 ± 1.7[c]	0.03 ± 0.003	6
10 pg/g ethinylestradiol	54 ± 3.0	5.3 ± 0.63	0.009 ± 0.0007	1.5 ± 0.19[b]	9	55 ± 4.7	19 ± 2.5	0.03 ± 0.003	7

[a] Results presented as mean ± SEM.
[b] Different from controls at $p < 0.001$.
[c] Different from controls at $p < 0.05$.

Table 3 Effect of *in ovo* exposure to atrazine on circulating concentrations of estradiol, progesterone, and testosterone in the 14-day male and female Japanese quail

	Circulating Reproductive Hormones: Male[a]				Circulating Reproductive Hormones: Female[a]			
Treatment	***n***	**Estradiol(pg/mL)**	**Testosterone(pg/mL)**	**Progesterone(pg/mL)**	***n***	**Estradiol(pg/mL)**	**Testosterone(pg/mL)**	**Progesterone(pg/mL)**
Control	13	< 25	429 ± 156	171 ± 14	4	< 25	65 ± 2.6	562 ± 243
123 ng/g atrazine	10	< 25	99 ± 16	393 ± 204	7	26 ± 1	91 ± 12	< 150[b]
246 ng/g atrazine	11	< 25	85 ± 16	241 ± 56	6	< 25	167 ± 94	300 ± 65
504 ng/g atrazine	13	< 25	112 ± 29	198 ± 24	6	< 25	67 ± 2.6	184 ± 28[b]

[a] Results presented as means ± SEM.
[b] Different from controls at $p < 0.05$.