Stages in Sublethal Exposures to EDCs in a Quail Model System

Grant Title and Principal Investigator

Stages in Sublethal Exposures to EDCs in a Quail Model System.

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Key Findings

- Japanese quail represent a sensitive and readily studied model for examining effects of EDCs on the endocrine and neuroendocrine systems of precocial avian species.
- The earlier the exposure of quail to EDCs, the more substantial and permanent the changes in measures of reproductive performance in adults and likely lifetime reproductive sucess.
- Hens can transfer lipophilic EDCs to their offspring through deposition in egg yolks.
- Components of the endocrine and neuroendocrine systems examined, including gonad weight and indicators of maturation, circulating sex hormone levels, and hypothalamic GnRH-I content and aromatase activity, were affected by the EDCs tested, depending on dose and timing of exposure.
- The most sensitive indicators of exposure to EDCs (i.e., effects shown consistently at the lowest doses) of the endpoints examined across all experiments included impaired reproductive behavior and delayed sexual maturation in both males and females.

Project Period: December 1997 to November 2000

Project Scope

Effects of endocrine-disrupting chemicals (EDCs) have been demonstrated in mammalian models, but less research is available for avian species. Precocial birds, such as quail, appear to be most sensitive to EDC effects during embryonic development. Although the Japanese quail (*Coturnix japonica*) is a nonnative lab species, its reproductive strategy is similar to that of many free-ranging precocial species. It is an excellent model for testing EDCs because its reproductive biology has been well characterized and because they reach sexual maturity in 8 weeks. Sublethal actions of selected EDCs (e.g., methoxychlor, vinclozolin) and the positive control estradiol benzoate (EB) were studied in this avian model to address several objectives:

- To determine which phases in the life cycle (embryonic, maturing, or adult) are most vulnerable to insult by EDCs;
- To determine if maternal exposures result in effects in offspring through transfer of EDCs or their metabolites into the eggs;
- To identify which components of the endocrine and neuroendocrine systems (e.g., gonads, hypothalamus-pituitary) are primary targets of the EDCs and their mode of action.

To accomplish these objectives, several experimental paradigms were employed: egg injection, maternal transfer studies, and multi-generation tests. Many endpoints were examined across the treatment paradigms, including those indicative of fitness, development and maturation, reproduction, behavioral responses, and endocrine and neuroendocrine functions of the hypothalamic-pituitary-gonadal (HPG) axis. In both birds and mammals, the hypothalamus, regulated by neurotransmitters and neuropeptides, synthesizes and secretes gonadotropin releasing hormone (GnRH-I). GnRH-I stimulates the pituitary gland to produce luteinizing hormone (LH) and follicle stimulating hormone (FSH), which in turn stimulate gonadal function, including ovulation in the female. Gonadal steroids (i.e., estrogen and testosterone) act in a feedback manner, primarily to the hypothalamic systems, but also to the pituitary gland. The GnRH-I system is sexually dimorphic in quail, with males having higher levels and greater release.

Project Results and Implications

Egg Injection Studies

Egg injection studies were used to assess the effects of estrogenic and antiandrogenic EDCs on embryonic development and subsequent sexual maturation and function of Japanese quail. Initial studies included injection with estradiol benzoate (EB) on incubation day 11 into groups of fertile quail eggs (20 μg/10 g egg, or 2 ppm EB in the egg); the negative controls included no treatment and injection of the corn oil vehicle only. In this project, known doses of three EDCs – methoxychlor, a weakly estrogenic organochlorine insecticide, vinclozolin, an antiandrogenic dicarboximide fungicide, and an estrogenic hydroxylated PCB (2’,4’,6’-trichloro-4-biphenylol) – were injected into groups of eggs on day 4 of incubation, a time just preceding both gonadal and brain sexual differentiation in the embryo (around days 3-4 and days 12-14, respectively). The same two negative controls were used. Vinclozolin was injected at doses of 25, 50, and 100 ppm in the egg, although delivery of the high dose was complicated by its failure to dissolve completely in the vehicle, and so those results are not reported in this document. Methoxyclor was injected at doses of 150 or 300 ppm in the egg, and the PCB was injected at low, medium, and high doses starting at 30 ppm. Some chicks were sacrificed from each group at hatching and examined for steroids and GnRH-I levels in the brain, while the remaining chicks were assessed for sexual function when they matured. In some cases, mated pairs from the same treatment group were evaluated for reproductive success.

Relevance to ORD’s Multi-Year Research Plan for EDCs

This project contributes directly to two of the three long-term goals of the ORD’s MYP for EDCs: (1) to provide a better understanding of the science underlying the effects, exposure, assessment, and management of endocrine disruptors, and (3) to support EPA's screening and testing program

This research focused on establishing neuroendocrine and endocrine-related targets of EDC modes of action at different stages of development in the Japanese quail (*Coturnix japonica*) model. In this study, four treatment paradigms were examined: egg injection, maternal transfer, one-, and two-generation dietary tests. Results from this study pinpointed several sensitive neuroendocrine, endocrine, and behavioral variables as endpoints for testing paradigms that were not part of current EPA or OECD toxicity testing guidelines for avian species. The time required to reach sexual maturity for both males and females and the vigor of male mating behaviors were particularly sensitive indicators of EDC exposure. Egg injection and the two-generation dietary tests provided the most complete and reliable data for establishing endocrine disruption in the quail model. In addition, the two-generation dietary testing paradigm provided the opportunity to assess the effects of EDCs at various life cycle stages at environmentally relevant exposure concentrations. Finally, this research is an important contribution to determining the validity of cross-class extrapolation for effects of EDCs, providing data on a precocial avian species that can be compared with mammalian models.

These experiments revealed reduced fertility and egg production in EB-treated pairs compared with both control groups. In particular, mounting behaviors of EB-treated males were dramatically reduced even though plasma androgen levels were unaffected; plasma estradiol levels were found to be increased. In EB-treated females, initiation of egg-laying was delayed 8 to 10 days and laying rate was reduced compared with controls (from 81 to 52 percent per day), although ovary weight was not affected.
Adult males hatched from eggs treated with 25 or 50 ppm vinclozolin mounted females less often than control males, and the same result was found for adult males hatched from eggs treated with both doses of methoxychlor. For males from eggs injected with the PCB, the latency to mount was significantly increased compared with controls. Thus, all three EDCs interfered with the development of normal male mating behavior in ways consistent with their classification as weakly estrogenic or antiandrogenic.

In the vinclozolin experiments, GnRH-I levels also were examined, revealing changes in GnRH-I levels in the brains of males but not females hatched from treated eggs compared with controls. Depending on location in the brain and the dose of vinclozolin, GnRH-I levels were either higher or lower than in birds from the no-treatment or vehicle-control groups, which also were significantly different from each other. The complicated pattern of changes seen suggests that the synthesis and release of GnRH-I might have been altered. However, these differences disappeared by adulthood. No differences were observed among groups in either hatchling or adult plasma estrogen or androgen levels or gonad weights.

Maternal Transfer Studies
Maternal transfer studies indicated that the compounds tested (i.e., methoxychlor, and the soy isoflavone genistein, a phytoestrogen) were readily maternally deposited and sequestered into the yolk. The chemicals were administered orally in gel capsules to groups of hens daily for five days and then the eggs were examined. After five days of administration of 50 or 100 mg genistein per hen, the compound was found in egg yolks in proportion to the administered dose (an average of approximately 2.5 µg genistein/egg yolk for the 100 mg dose group and 1.25 µg genistein/egg yolk for the 50 mg group, see Figure 1), although the quantities are relatively low considering the administered dose (up to 100 mg genistein daily). Similarly, administration of 2.5 mg methoxychlor in capsules to hens daily for five days resulted in significant transfer of the compound to the egg yolks.

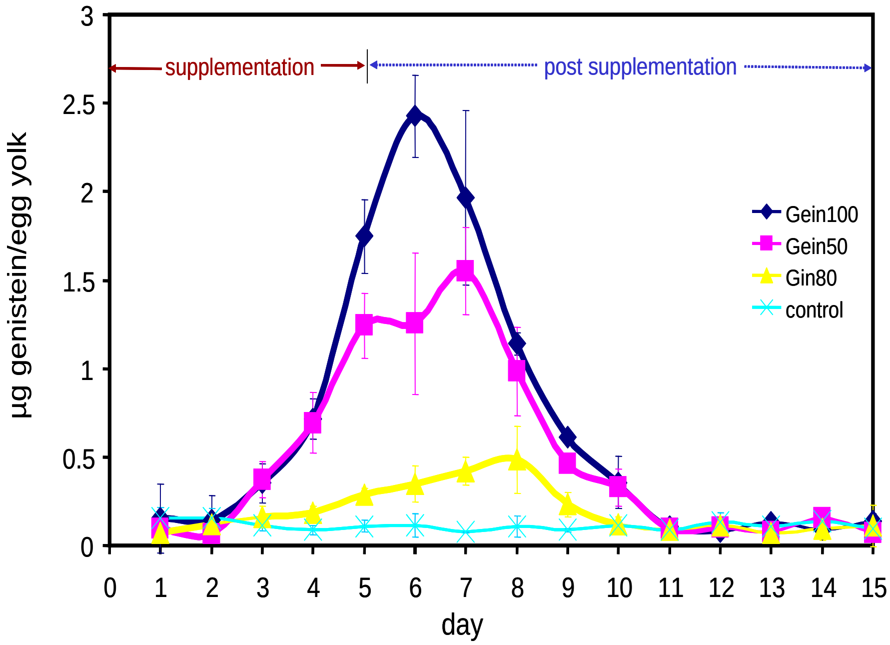

Figure 1. Figure 5: Maternal transfer of soy isoflavones into the yolk; no transfer was found to albumin.

For comparison, endogenous steroid hormone levels in the yolks of eggs from untreated hens also were examined. These control eggs revealed that both androgen and estradiol are measurable even during the first week of incubation. Androgen levels were highest (20 ng/g) on day 3 and lowest (3.8 ng/g) on day 7 of incubation. Estradiol was highest (8.1 ng/g) on day 3 and lowest (0.21) on day 5 of incubation. During the last half of incubation, the steroid levels in the circulating plasma of the embryo reflected the levels of hormone in the yolks. That implies that lipophilic EDCs, which can transfer from hens to their eggs where they are sequestered in the yolks, can be taken up by the embryos as the yolk is used during development.

Multi-generation Dietary Exposure Studies
Multi-generation experiments were conducted with methoxychlor at 0, 0.5, or 5, and at 0, 5, or 10 ppm in the diet to assess potential effects of field-relevant dietary exposure levels. The parental generation (P1) was exposed for 8 weeks starting at age 13 weeks, with eggs collected for the next generation at weeks 3 and 4 of the exposure period. P1 birds were examined for plasma steroid hormones and assessed for

number of eggs laid per hen, shell strength, egg production, egg fertility, fecal estrogen and androgen levels, and brain neurochemicals at 21 weeks of age. The F1 generation was exposed to the same dietary exposure regimen as their parents, with some F1 birds sampled at 8 weeks (early puberty) and others sampled at 29 weeks after producing eggs that comprised the F2 generation. All F2 generation birds were fed the control diet and were sampled at 11 weeks of age. The same endpoints were examined.

A number of the endpoints examined appear to be potentially reliable indices of low-level EDC exposure. Among these endpoints, plasma steroid hormones were reduced in both males and females of the P1, F1, and F2 generation birds compared with controls. In P1 birds, none of the measures of reproductive performance differed from controls; however, plasma steroid levels were affected, and there was an indication of impact to hypothalamic catecholamines. Steroid hormones were reduced and less variable in treated F1 and F2 pubertal males and females than in controls of the same age, possibly due to delayed maturation of both sexes. Birds exposed to higher concentrations of methoxychlor in their diet exhibited higher rate of reproductive failure (defined as lower viability of chicks and a higher proportion of birds that did not become reproductive), which was reflected in their lower plasma steroid hormone levels. Delayed maturation and lower plasma steroid hormones was accompanied by impaired sexual behavior in males and more variable responses related to fertility, egg production, and body weight in females. This variability was due in part to individual differences in response to the pesticide exposure, with some birds maturing at a normal rate and others showing delayed onset of mating behaviors or egg production. Neurochemical measures, including hypothalamic catecholamine concentrations and aromatase activity, revealed similar dose-related changes the P1, F1, and F2 groups examined, suggesting that these neuroendocrine modulators of reproduction are important targets of EDCs.

Implications

The series of experiments described above indicate that embryos represent the most vulnerable life stage of the quail, while adults represent the least vulnerable. These and subsequent experiments have indicated that maturing birds are intermediate in sensitivity, as expected. Although birds first exposed as adults (P1 birds in the dietary experiments) exhibited some effects (e.g., lower plasma steroid hormones), fertility, egg production, and hatching success were unaffected. However, hens deposit these chemicals into their eggs, as shown in the experiments in which the eggs from hens administered methoxychlor or genistein showed dose-related increases in the concentrations of those chemicals in the yolks. Thus, effects on subsequent generations can be expected, as indicated by the effects demonstrated in birds hatched from eggs from parents ingesting the chemical in the diet. And, if exposure continues in subsequent generations, additional effects can be expected as shown in the two-generation experiments with methoxyclor.

Several measures proved to be sensitive indicators of EDC exposure in the quail model. The latency to sexual maturation in males and females (as measured by the size and condition of testes and ovaries and by mating behaviors) and the vigor of male mating attempts were sensitive indicators of EDC exposure. Although sensitive to EDC exposure, plasma and fecal sex hormone levels were more variable across individual birds. Traditional measures of reproductive fitness (e.g., fertility, egg production) were less sensitive indicators. Of the treatment paradigms, egg injection and the two-generation dietary testing design provided the most complete and reliable data. These data are important for establishing reliable and sensitive measurement endpoints and study protocols for detecting endocrine disruption in avian species. Furthermore, the two-generation testing paradigm used in this study can be used to assess the effects of EDCs at different life-cycle stages.

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NCER Project Abstract and Reports:
http://cfpub2.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/163/report/0