

Neuroendocrine and behavioral consequences of embryonic exposure to endocrine disrupting chemicals

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Introduction

Endocrine disrupting chemicals (EDCs) have been defined functionally due to their activity on endocrine systems. Much of the attention given to these chemicals arose from the recognition that prenatal exposure to diethylstilbestrol (DES) had long term consequences in women. As more information was collected, it became clear that EDCs had the potential to impact animals across many phyla (8, 9, 10, 38). Furthermore, the exposure of wildlife at field relevant exposures is often at sublethal concentrations, making detection of effects of EDCs challenging, especially when attempting to separate other interacting factors in the animal's environment. These additional factors may include environmental conditions, food availability, disease, or confounds such as simultaneous exposure to several chemicals. It is therefore important to develop reliable and sensitive measurement end points that are appropriate for the endocrine or organ system that is the target of the EDC. A number of workshops and symposia have addressed these issues, generating publications and reports that raised awareness to the potential effects of EDCs on wildlife and humans (15, 23, 32). These and other publications have also provided a summary of the data available on the impact of EDCs in wild populations and the incidence of exposure (15, 4, 14). In 1998, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) submitted a report that recommended a tiered approach to testing suspected EDCs, which involved screening followed by selected bioassays, and subsequent single and

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multigenerational tests when indicated. In birds, a two-generation testing paradigm was selected for evaluation. Development and validation of these testing paradigms have continued (see Endocrine Disruptor Screening Program:

(http://www.epa.gov/oppfead1/cb/csb_page/updates/endocrin.htm) for progress in the area of regulatory activities and for updates on the status of validation of specific tests. A parallel activity has been ongoing through the Organization for Economic Co-operation and Development, which established a Working Group on Avian Reproduction Toxicity Testing. This group has been considering the testing parameters and measurement end points that are reliable and sensitive indices of EDC exposure in birds and generated a discussion document (6). This Working Group has revised the Avian Reproduction Toxicity Test guideline, which is under review and is drafting a protocol for a Multigenerational Reproduction Toxicity Test guideline, to be reviewed by the OECD member nations.

In birds and mammals, early attention had been focused on the estrogenic activity of many of the environmental chemicals (30). Recognition that many of the suspected EDCs were likely to have estrogenic activity also led to development of *in vitro* screens for rapid detection of these chemicals (4). Simultaneously, there was growing concern about the effects of environmental chemicals of species living near water, such as the Great Lakes or Chesapeake Bay, resulting in biomagnification of chemicals for fish eating birds (25, 43). Because some of the EDCs also interact with the thyroid and adrenal endocrine systems, they have the ability to affect metabolic endocrine responses, as well as reaction to stressors and immune function. Overall, this field has a great deal of complexity due to the number of EDCs in the environment, temporal and spatial variation in somewhat sporadic distribution of specific EDCs, and their potential interaction with other contaminants. Fortunately, there are several databases available that integrate the available data into an accessible format for determining potential risk for populations in selected regions (for example, see 44). Some of the basic issues and an overview of the data collected in birds is discussed below. Clearly this is an area that requires an integrated approach amongst avian biologists that are working in the areas of endocrinology, neurobiology, field biology, and toxicology in order to address these questions in a responsible and scientific manner.

Issues associated with the identification and regulation of EDCs in avian species

Relative potency and effects compared to other environmental factors

A major confound in the issue of EDC effects in birds is distinguishing the impact of other environmental and health impacts from those associated with EDC exposure. In fact, it is likely that these factors all interact with one another to affect the overall viability and fitness of the individual. Furthermore, unless a site is highly contaminated in a homogeneous manner, it is likely that an individual may not be consistently exposed to EDCs. Therefore, it is difficult to clearly assess the impact of EDCs in the field because the level of exposure is often variable and the bird's contact with the EDC(s) may be inconsistent according to the distribution of the compounds in their habitat. Therefore, in order to evaluate EDC impact for the purpose of risk assessment, it is critical to determine the availability of the EDCs for species that are likely to be exposed and if there are metals and other contaminants in the environment that will also affect the birds (19). Mode of exposure is also an issue, especially when considering sensitive stages in the life cycle. For example, pesticides sprayed in an orchard are likely to coat eggs in nests located in the trees that are sprayed. Moreover, contaminants deposited on the fur or feathers may be ingested during grooming or occur on/in prey items that are ingested or fed to the young. These embryos are then exposed to EDCs during a critical time in development. However, passerines appear to be less sensitive to sublethal doses of EDCs during embryonic development compared to precocial species potentially due to the timing and mechanisms of sexual differentiation as well as to the mode of exposure to EDCs associated with diet and habitat (2,34, 35, 38). Therefore, these factors must all be considered, especially when considering the risk of EDCs to field species. Clearly, more characterization of the effects of EDCs to precocial and altricial must be available along with the pharmacokinetics of the EDCs in the environment in order to generate realistic risk assessment models. Finally, these models must consider avian biology rather than being based on mammalian, fish, or invertebrate models because of phylogenetic differences in metabolism, reproductive strategies, habitat considerations, and life history.

Regulatory issues and risk assessment

Toxicity testing with end points that detect endocrine disruption

A basic issue is the development of an appropriate testing paradigm, including reliable end points. One source of confusion has been that avian toxicology tests currently in use generally include reproductive end points as a measure of the fitness of the adults. Because these

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reproductive end points, such as fertility and hatching success, are included in the testing paradigm, it has been assumed that these measures are sufficient. However, these reproductive end points may not necessarily be appropriate, reliable, or sensitive for detecting an endocrine disrupting chemical. Therefore, it is important to distinguish the toxicology test from a test designed for detecting an endocrine disrupting chemical. A number of studies are underway in laboratories around the world; many of these laboratories have participation in the OECD Expert Group on Avian Reproduction Testing. At this time, the measurement end points for consideration in a testing paradigm include neuroendocrine, behavioral, endocrine, and whole organism measures which relate to the health of the bird. Deliberations continue and information and progress in revisions, including consideration of EDC impact in the testing paradigms used in preparing applications for registration of pesticides and other chemicals for agricultural or residential may be found at the following website:

<http://www.epa.gov/pesticides/regulating/index.htm>

Avian species selected for monitoring and testing

In most of the toxicological applications for regulatory monitoring, species indigenous to the United States have been used for testing. This means that the species of choice have been the mallard duck and northern bobwhite quail (6, 37). Although the toxicological responses of these species have been tested with a variety of compounds, few background or baseline data exist for the reproductive endocrinology of these birds especially relative to EDC impact. Conversely, there are data available on the reproductive and metabolic endocrinology and on the neural regulation of reproductive behavior in Japanese quail, zebra finches, and doves. However, these species were not considered for toxicological testing until recently. Japanese quail have been under intense study recently with the recognition that multigenerational approaches are necessary to detect some of the more subtle effects of EDCs. Moreover, since field exposures are often at low levels or in an unpredictable frequency, the impact may be more significant over several generations. As such, multigenerational testing is necessary to detect this type of low exposure, long term outcome. In order to do multigeneration tests, slowly maturing species, including northern bobwhite quail and mallard ducks require long term studies. Our experience in conducting a two-generation bobwhite quail dietary study was that, beginning with hatching the parent generation, at least 24 months was required to complete the in life portion of the study. This becomes a prohibitive time frame for use in regulatory applications.

Baseline on Japanese quail neuroendocrinology and rationale for choice of endpoints used in studies discussed below

Studies in Japanese quail take advantage of a great deal of information available on the biology, neurobiology, and endocrinology of this species. In addition, Japanese quail have a short incubation period (17 days), mature quickly (8 weeks), and have a long peak production period (3-12 months). EDCs impact development and sexual differentiation in Japanese quail because they are hormonally active at a time that native estrogens and androgens exert long-term effects on endocrine and behavioral responses (7, 22). Further, estradiol is a critical element in sexual differentiation in quail. During embryonic development, females have high relative concentrations of estradiol/androgen whereas males have low relative estradiol/androgen. Moreover, there are sex related differences in the patterns of changes in these steroids. In females embryos, plasma E₂ rose until hatch and decreased, post hatch (34, 35). In males, plasma androgen peaked at embryonic days 14-17 (E14-E17) and declined post hatch (34). These steroid hormones are primarily produced by the embryonic gonads, with some contribution from the adrenal glands (1). In addition, yolk steroid hormone content reflects embryonic steroid hormone levels, suggesting that the yolk serves as a hormone depot for the embryo (Abdelnabi and Ottinger, unpublished data). This developmental pattern allows for precise experimental manipulations at timed phases in ontogeny as well as selection for suitable sampling paradigms that can be used for range finding tests and other manipulations.

Sex differences were observed in hypothalamic catecholamine and indolamine concentrations in untreated quail hatchlings (1, 34, 35). Females had higher levels of norepinephrine (NE) than males over embryonic development, whereas males had higher dopamine (DA) levels (34, 35). Our current data provide evidence that estrogenic EDC exposure can alter these monoamines in hatchling and adult quail, suggesting potential consequences for neuroendocrine regulation of reproductive endocrine responses.

Finally, maternal transfer of steroids is a likely mode of embryonic exposure; female quail given E₂ implant transferred estradiol to offspring via the yolk (3). Therefore, embryonic steroid exposure from endogenous and exogenous sources appears to be critical in the sexual differentiation of hypothalamic neurotransmitter systems (35[MAO5]). Altering the relative exposure to the gonadal steroids, even with weakly hormonally active compounds therefore has the potential to affect sexual differentiation if exposure occurs during sensitive periods in development.

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Testing paradigms and measurement end points

Chemicals chosen for testing in our laboratories and effects of these chemicals

Estrogenic EDCs

Estradiol benzoate: Administration of exogenous gonadal steroids to embryonic quail alters sexual differentiation, resulting in abnormalities in the female reproductive tract and impaired male sexual behavior (3, 45). The action of estradiol may differ in males and females in that both have sex-specific endogenous steroid hormone patterns during embryonic development; specifically males have relatively higher androgen to estradiol levels and vice versa in females (34). Relatively low concentrations (20 µg/egg estradiol benzoate or 2 ppm estradiol) injected at embryonic day 11 (ED 11), decreased male sexual behavior and decreased egg production and fertility in females (35). Fifty µg/egg (5ppm estradiol) at ED 11 caused uterine prolapse, egg bound females and low egg production, while 500 µg/egg (50ppm) estradiol at ED 11 caused uterine prolapse, egg bound females and low egg production, while 500 µg/egg (50ppm estradiol) at ED 11 resulted in no egg production and both 50 and 500 µg/egg caused persistent right oviducts (Ottinger et al., unpublished data).

Methoxychlor (MXC): The deleterious effects of EDCs have long been recognized in wildlife, with effects of DDT on the avian egg shell as one such example. Methoxychlor is an organochlorine pesticide and was developed to replace DDT. It is used as a pesticide for fruit, vegetables, trees, home gardens, forage crops, and livestock. Highest intensity of use is in the northwest and in eastern seaboard states, with concern for human exposure (17, 36). In mammals, MXC has been linked to reproductive dysfunction.

In laboratory experiments MXC was maternally transferred into eggs when hens were fed 2.5 mg/day in a capsule over five days (Ottinger et al. unpublished data). Exposure of Japanese quail embryos to 1.5 or 3.0 mg/egg MXC resulted in a significant increase in gonadotropin-releasing hormone-I (GnRH-I) in female hatchlings and a significant decrease of GnRH-I in female adults (39). Adult males exposed to 1.5 or 3.0 mg/egg MXC had less sexual behavior (35). Exposure of Japanese quail at ED 0 to 5 mg/egg MXC decreased or completely inhibited adult male sexual behavior (12). Therefore, if embryonic exposure to MXC has negative effects on female fitness, also, then hatchling GnRH-I will be a sensitive biomarker.

Soy Phytoestrogens: The phytoestrogens in soy, especially genistein are hormonally active. The primary biologically active isoflavone in soybeans is genistein, which is approximately 1000 times less active than estradiol *in vitro* (13, 46, 26, 47). The phytoestrogens are

found primarily in the soy protein, which in feeds, may have relatively high levels of genistein or other phytoestrogens in their aglycone, glycosylated, and acylated forms (27). Interestingly we also found that of the phytoestrogens in soy, genistein was the primary isoflavone transferred into the yolks when fed to quail hens in a dose dependent manner. Therefore, the soy protein content in feeds is noteworthy as there is the potential for it provides evidence that this biologically active phytoestrogen is a potential for maternal deposition into the yolk and subsequent effects on the developing embryo.

Studies in which quail eggs were injected with 10, 100, or 1000 µg of genistein showed that this phytoestrogen has bioactivity in birds. Results showed that the highest dose of genistein inhibited sexual behavior in adult males (Panzica et al., unpublished data). Because of the association of vasotocin and male sexual behavior, this system was further investigated in phytoestrogen treated males (40). Phytoestrogen treated males had less vasotocin-immunoreactive cells stained in areas that regulate reproductive behavior, suggesting that the genistein may have interfered with sexual differentiation of male behavior. It is intriguing to note that there is evidence from mammalian studies that phytoestrogens affected aromatase enzyme (41). Therefore, it is possible that the EDCs act via several ways to impact reproductive endocrine and behavioral responses.

Androgen or thyroid active EDCs

Vinclozolin: Vinclozolin is a fungicide that blocks androgen receptors (24, 21). In quail, we found that egg injections with vinclozolin at relatively low levels resulted reduced male sexual behavior and GnRH-I in hatchlings (29).

p,p'-DDE: DDE is a persistent, bioaccumulative environmental contaminant, exposure to which was associated with egg shell thinning in many bird species. Although DDT (the parent compound of DDE) is no longer used in the United States and the European Union, DDE is persistent in many ecosystems and is a chemical of concern in many risk assessments. Exposure of Japanese quail embryos to 40 µg/egg DDE injected at embryonic day 4 decreased male sexual behavior and early onset of puberty in females, but did not affect hatchability compared to controls. Egg injection with DDE (low=20µg/egg and high=40 µg/egg) also impacted reproductive end points. Females showed delayed sexual maturation, at the higher dose, and males treated with the higher dose showed impaired mating behavior ($p < 0.05$; Quinn and Ottinger, unpublished data).

Polychlorinated biphenyls (PCBs): PCBs have been the subject of intense investigation for many years as they have been identified as

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contaminants of concern, especially in fish eating birds (25, 28, 32, 48). The PCBs are especially complex due to the number in the environment and their persistence. Further, depending on the structure of the compound, there are estrogenic, androgen, thyroidgenic, or a combination of these effects from PCBs. PCBs are also a problem for carnivorous birds due to relatively higher concentrations in the fatty tissue of their prey, which resulted in decreased thyroxine and estradiol levels in species such as the kestrel (42). Studies conducted in our laboratory with a primarily estrogenic PCB have shown that fertility and reproductive performance were reduced by PCB exposure during embryonic development (see Table 1). As with many of the pesticides, sexual behavior proved to be a sensitive index of embryonic exposure in Japanese quail.

Table 1. Exposure at E4 by egg injection resulted in decreased reproductive performance (fertility and egg production), due in part to reduced male reproductive behavior (Henry *et al.*, unpublished data).

| Generation | Treatment | n | Latency (sec) | Attempts | Cloacal Contacts |
|------------|-----------|----|---------------|------------|------------------|
| P1 | Control | 13 | 35 ± 10.05 | 5.5 ± 1.98 | 2.16 ± 0.45 |
| | PCB | 8 | 77.8 ± 19.45 | 5.9 ± 0.97 | 2.0 ± 0.44 |
| F1 | Control | 11 | 42.6 ± 14.44 | 8.4 ± 1.95 | 2.3 ± 0.47 |
| | PCB | 6 | 107.0 ± 14.97 | 2.8 ± 0.82 | 0.3 ± 0.20 |
| F2 | Control | 16 | 49.8 ± 9.96 | 6.0 ± 0.86 | 2.2 ± 4.0 |
| | PCB | 12 | 77.0 ± 13.25 | 5.3 ± 2.6 | 0.9 ± 0.33 |

PCB = 2',4',6 -trichloro-4-biphenylol

P1 = parent generation

F1 = first generation

F2 = second generation

n = number of tested males/day

Effects of EDCs in birds and validating appropriate measures

Population level effects: measurement end points and assessment end points

Ultimately, it is critical to ascertain the potential of EDCs to affect wild birds, especially at a population level. As discussed above, many of the mechanisms that regulate reproductive endocrine and behavioral responses have been extensively investigated in both laboratory models and in wild birds, particularly those that are likely to be impacted by EDCs. Once the appropriate measurement end points have been established, they very often are also used as assessment endpoints to determine population level effects.

Measurement end points: reliability and use in laboratory studies:

Fitness end points: provide an indication of the individual's fitness, including health, reproduction, and vigor. These measures are crucial to survival and production of offspring, but may underestimate long-term or transgenerational effects of the EDCs. Our studies have shown that it takes relatively high doses of weakly estrogenic compounds to exert measurable effects on these types of end points. Conversely, some EDCs also have toxicological effects and will be detected in both toxicology tests as well as tests designed for detecting EDCs. These compounds present less of an issue due to their potency. As such, regulatory decisions and risk assessment for these compounds are much less complicated.

Endocrine, behavioral, and neuroendocrine end points: As mentioned earlier, a primary route of exposure is via maternal deposition of the EDC into the yolk (3). This is the case for methoxychlor as well as for genistein and presumably other lipid soluble chemicals. Although EDCs are generally weaker in action than endogenous steroids, including exogenous estradiol, the estrogenic pesticide methoxychlor (MXC) slowed sexual maturation in both males and females. Treated males also had impaired sexual behavior, similar to the effects of embryonic estradiol (35).

Similarly, androgenic EDCs, such as vinclozolin, a fungicide that blocks androgen receptors also affected GnRH in hatchlings and sexual behavior in adult males that had been exposed as chicks (5). These adults also showed slower sexual maturation and impaired behavior during maturation. Although in many cases, the birds appear to "catch up", a critical question would be the impact of delayed maturation and impaired sexual behavior for bird populations in the field.

Therefore, in our studies male sexual behavior has consistently been a sensitive and responsive measurement end point. Aromatase enzyme has been clearly shown to modulate male sexual behavior (5, 11). However, in our studies, we have not observed a dose dependent effect of embryonic EDCs on aromatase enzyme activity in adults. Arginine vasotocin (VT), which is localized in brain regions that modulate reproductive behavior is also sexually dimorphic and responsive to gonadal steroids (40). In fact VT may prove to be a more sensitive dose dependent index of EDC effects. This is based on the findings from embryonic genistein exposure in which a dose dependent decrease occurred in immunostaining that correlated with the loss of male sexual behavior (Panzica et al., unpublished data).

Immune end points: Administration of many different types of androgens, including androsterone, androstene-3, 17-dione,

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methylandrostene diol, 5α -dihydrotestosterone (5α -DHT), testosterone propionate (TP), and 19-nortestosterone, disrupts embryonic development of the bursa (18, 33). DHT treatments resulted in reduced numbers of proliferating bursal cells in broiler chicks (31). Bursas from chicken embryos treated with TP at E 3 were considerably smaller than controls on E 10 (33).

Some laboratory studies have shown specific immunotoxic effects of individual EDCs. Planar halogenated aromatic hydrocarbons, such as polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin have been shown to have immunotoxic effects (20), including developmental effects on the bursa. PCB 126 caused decreases in bursa weight and numbers of developing B lymphocytes in the bursas of chicken embryos (16). PCBs and dioxins have also been linked to thymic atrophy and suppression of mitogen-induced proliferation, mixed lymphocyte responses, cell-mediated cytotoxicity, and humoral responses (reviewed in 20).

Interpretation of field studies is difficult when trying to establish links between individual chemicals and individual effects. Organisms are most often exposed to multiple types of EDCs at any one period of time in the environment. Exposure to multiple EDCs makes it impossible to link any immunotoxic effect to any one chemical. Further, many of these studies are observational, and as such, causal relationships cannot be established. The matter is further complicated by the fact that many of the mechanisms behind EDC-induced immunosuppression remain unknown.

Concluding remarks

The issue of EDCs and their impact on avian species has gained visibility in recent years due the evidence of endocrine activity. The extent of hormone-like activity is the subject of extensive investigation. Results from these studies will provide the basis for regulatory guidelines and tests that should serve to protect field birds from exposure to deleterious levels of EDCs. It is not clear, however, if EDCs interact with other contaminants or predispose an individual to effects from other environmental challenges or disease. As such, more information on the extent of EDCs impact and stages of the life cycle that are most sensitive will be extremely useful. Furthermore, the traditional toxicological tests, even those including reproductive end points may not be sufficient to reliably detect and characterize the effects of EDCs. It is important to establish reliable indices that are appropriate for the mode and target of action for each class of EDC and for regulation to be based upon these tests.

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