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Assessing the consequences of the pesticide methoxychlor: neuroendocrine and behavioral measures as indicators of biological impact of an estrogenic environmental chemical

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Abstract

Japanese quail provide an advantageous avian model for assessing long-term biological consequences of endocrine disrupting chemicals (EDCs). These studies examined route of exposure and vulnerability to biological impact of EDCs over the life cycle in a precocial avian model, the Japanese quail. Embryonic exposure occurs with maternal deposition and methoxychlor (MXC) accumulated with maternal exposure. Egg injections of MXC or estradiol at selected stages of development impacted hypothalamic neuroendocrine systems in hatchlings and affected sexual maturation, with evidence for long-term effects on neurotransmitters and male behavior. Two-generation dietary studies were conducted to examine transgenerational effects of EDCs. Adult quail (P1) were exposed to dietary MXC (0, 0.5 and 5 ppm), with continued exposure in their offspring (F1), and control diet for all F2 chicks. Toxicological end points, including fertility, hatching success, and 14-day viability were unaffected. F1 and F2 male offspring from MXC-treated pairs MXC had impaired mating behavior and altered plasma hormones. These studies confirm neuroendocrine and behavioral measures as reliable indices of exposure to an estrogenic EDC. Moreover, maternal deposition remains a primary route of EDC exposure, with potential deleterious consequences for field birds, especially precocial species that appear to be particularly sensitive to embryonic EDC exposure.

Keywords: Neuroendocrine systems; Sexual behavior; Estrogens; Japanese quail

1. Introduction

Endocrine disrupting chemicals (EDCs) include a number of environmental chemicals and chemicals found in plant products that interact with an endocrine system, often due to activity as a hormonal mimic [43]. Unfortunately, it was not apparent until relatively recently that pesticides, herbicides, industrial chemicals and even plant hormones have endocrine activity in vertebrate species. Recognition of the potential impact that these compounds may have on wild bird populations has stimulated research to characterize the effects of these chemicals and consideration of phylogenetic variation in biological response to exposure (for review, see [30]). Once reliable end points have been identified and characterized, this will permit non-invasive assessment of EDCs in the environment [14,15].

Given our current understanding of the activity of EDCs, many of the traditional measures for toxicity of compounds appear to be relatively insensitive in birds as well as in other species. These measures have included overall health and food intake, fertility, viability of offspring, gross morphology of gonads and general measures of growth and development. Current toxicity testing has not included endocrine specific

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variables such as plasma steroid hormones, reproductive neuroendocrine or behavioral end-points. It is critical to establish testing methods that utilize meaningful variables, and quantitatively consider relative change in these end-points, which are responsive to EDCs and reliable indicators of biological impact. In total, the test methods for EDCs should include overall measures of reproduction, such as fertility, viability and number of chicks etc., as well as additional end-points indicative of development and function of the hypothalamic pituitary gonadal (HPG) axis. These additional measures will provide sensitive and reliable indices of the potential for a compound to have endocrine disrupting activity.

The critical role of steroid hormones has been demonstrated in embryonic development and sexual differentiation as well as during sexual maturation, with many studies focused on the neural mechanisms involved in modulation of endocrine and behavioral components of reproduction [3,7,12,20,25–27,45]. Administration of exogenous gonadal steroids altered sexual differentiation, resulting in sex differences in later male or female behavior [2,3,7,31]. In quail, the timing of embryonic exposure to testosterone, E2, fadrozole or tamoxifen by embryonic day (E12) altered later expression of sexual behavior; defeminized females [7,30,32,33]. In addition, early steroid exposure also alters gonadal development, with fadrozole and tamoxifen exposure producing defeminization of the ovary and accessory structures [9,12,16,32]. Finally, female quail given E2 implants were found to transfer estradiol to offspring via the yolk [4]. This provides further evidence that maternal deposition of lipophilic hormones or EDCs are a likely pathway for exposure in field species.

Methoxychlor (MXC) is a widely used pesticide that gained in popularity after the ban on DDT in 1972. In rats, MXC clearly impacts both male and female embryos [6,13,17,41]. There is also evidence for imprinting leading to altered adult sensitivity to EDCs [35]. Furthermore, it has been demonstrated that while MXC itself may be short-lived, its metabolites are also biologically active and these compounds may imprint embryonic tissues, resulting in altered responses in adults [5,6]. Therefore, MXC is considered a pesticide with the capacity to induce endocrine disruption, in the form of the parent compound as well as its metabolites.

Because of its wide usage, there is a great deal of potential for exposure to MXC in the field for birds and impact at the individual and population levels. This pesticide has long been considered safe for use because of its short half-life; however, most of these early tests on MXC were conducted on adult or near adult birds. Given the estrogenic nature of MXC, it is reasonable to suspect that even short-term maternal exposure could transfer to the egg/embryo and have long-term effects. A bioassay has been developed in the zebra finch that will prove useful to assess specific responses in passerine [20]. Studies in chickens and Japanese quail have shown impact of EDC exposure during embryonic and adult phases of the life cycle [9,13,16,18,23,28,29,31,37,38]. We will discuss our findings in the Japanese quail in more detail below, concentrating on the estrogenic pesticide, MXC, and its positive control, 17-β estradiol.

2. The Japanese quail model

Previous studies have shown that the male Japanese quail is exquisitely sensitive to the effects of exogenous estradiol especially during critical periods of sexual differentiation of neural systems. In addition, the hormonal basis for sexual differentiation in avian species differs from mammals, thereby making mammalian tests potentially inadequate for assessing EDC impact on avian species. Regulatory agencies have routinely used avian species indigenous to North America for testing toxic chemicals for industrial and agricultural applications. These species have included mallard ducks and northern bobwhite quail. However, there are relatively few data available on the HPG axis in these species. Conversely, a great deal is known about neuroendocrine regulation of reproduction in Japanese quail, but few data have been collected on effects of EDCs on neuroendocrine systems in quail. Therefore, our research has focused on investigating the impact of EDCs in the Japanese quail as a model for precocial avian species and determining end-points that provide reliable and sensitive indices of endocrine disruption.

The avian model has a distinct advantage over mammalian models of embryonic neuroendocrine differentiation. The maternal contribution to the egg is limited to the period between formation and oviposition [9,10,39]. Researchers can thereafter manipulate embryo conditions in a controlled environment to better understand development. It thus provides a convenient model in which to study the effects of chemical exposure to the embryo.

Further, a great deal of information is available about the role of steroid hormones in embryonic development and maturation of the Japanese quail, and the mechanisms involved in these processes [3,7,21,22,25–27]. Measurement of endogenous steroids during embryonic development has shown that both adrenal and gonadal steroids contribute to circulating hormone levels. In the female embryo, plasma E2 rose until hatch and decreased, posthatch [1,24,29]. Increased 5β reductase enzyme activity was found in the brain of male quail embryos between E7 and E15, possibly acting to protect males from being behaviorally demasculinized by inactivating testosterone [8]. In males, plasma androgen peaked at E14–E17, and declined post-hatch [24]. A similar embryonic pattern in plasma gonadal steroids, including an adrenal contribution has also been documented in the domestic chicken [21,36]. Females treated with estradiol at E10 or E12 had higher preoptic dopamine (DA) content as adults and treated males had relatively lower preoptic norepinephrine (NE) content and were behaviorally demasculinized. The preoptic-lateral septal region (POA-SL) is also an important area because it contains many of the gonadotropin releasing hormone-I (GnRH-I) cell bodies and regulates copulatory behavior, while the median eminence (ME) is the site of GnRH-I release. Finally, neurotransmitters, including

NE, epinephrine (EPI), DA, and serotonin (5-HT) modulate both endocrine and behavioral responses in the Japanese quail [2,30].

Bobwhite quails are indigenous to the United States and have historically been the preferred as well as regulatory model for testing the toxicity of chemicals to be used in the environment. They are not, however, the ideal laboratory species. With regard to animal care, Bobwhite quail can be easily stressed and do not reach sexual maturity until approximately 6 months of age. Perhaps most frustrating is the fact that little basic research has been performed in Bobwhite quail. Thus, drawing conclusions about the effects of specific chemicals in these animals can be difficult.

The Japanese quail has been extensively studied over the years. The small size and rapid growth of this species also permit the completion of multi-generational studies in less than one year. Furthermore, our results (unpublished) demonstrate that Japanese quails respond to EDCs in a similar manner to Bobwhite quails under laboratory conditions. These data suggest that we can use the more compliant species, the Japanese quail, to perform studies that are biologically and environmentally relevant to assess the potential impact of suspected EDCs on field populations of birds.

3. Studies in Japanese quail

3.1. Embryonic exposure to estradiol has long-term consequences

3.1.1. Maternal transfer of estradiol from hen to egg

The early developmental stages in the formation of the oocyte occur prior to ovulation and are similar across a number of avian species [10]. With recruitment of the primary follicles into the ovulatory hierarchy, follicular development is accompanied by yolk deposition. Following ovulation, albumen encircles the yolky oocyte as it traverses the oviduct. Lipophilic compounds appear to concentrate in the yolk portion of the egg and provide the opportunity for maternal deposition of steroid hormones and EDCs. In wild birds, there is increasing evidence that the maternally deposited steroid hormones become an important factor for later individual behavior [39]. In quail hens, daily injections of estradiol or an estradiol implant resulted in higher concentrations of circulating estradiol as well as increased yolk content of estradiol [4]. The presence of in ovo estradiol at critical periods in embryonic development has resulted in impaired sexual behavior in male birds [3,7,13,30]. Therefore, exposure of the hen to an EDC that is likely to transfer into the yolk would become a chemical that is not only available to the embryo, but is likely to impact developmental processes throughout critical phases in ontogeny.

3.1.2. Effects of estradiol egg injections on neuroendocrine systems and sexual behavior

We conducted experiments using egg injection to study the effects of estradiol dose and timing on embryo maturation in both one- and two-generation paradigm. As discussed above, these experiments have the advantage of a great deal of previous and ongoing research on the mechanisms involved in the sexual differentiation of birds [3,7,16,20,22]. Therefore, it has been established that gonadal differentiation is complete by E4 and the HPG axis is functional after E14, similar to the domestic chicken ([42,44] for reviews, see [30,36]). Because we wished to affect sexual differentiation of sexual behavior and hypothalamic systems, Japanese quail embryos were treated at E11 (17 day of embryonic development) with estradiol benzoate (EB; 20 μg/egg). This treatment was designed to provide data as a positive control to subsequent experiments using the more weakly hormonally active EDCs. Therefore, a relatively low dose of EB was used as compared to some of the higher levels used in previous studies, with the intent of partially interfering with sexual differentiation of the male more similar to weakly estrogenic EDCs [2,3,7,28,33,38]. This study also differed from behavioral neurobiological studies in that the offspring of treated parents also were treated embryonically with EB in a two-generation paradigm. Females and males of the same treatment were paired to determine effects of EB exposure on fertility. Results showed that embryonic EB treatment resulted in significant reductions ($p \leq 0.05$) in hen day production (90.2% versus 54.1%; control versus EB) and fertility (85.3% versus 33.4%, control versus EB; [22,27]). The females that were EB-treated were also slower to reach sexual maturity compared to oil-treated controls, as monitored by the age at which 50% of the hens had initiated egg production. Fifty percent of the control females were reproductive by 58 days of age, whereas 50% of the EB-treated hens achieved egg production at 74 days of age. This delay appeared to be associated with some direct ovarian effects of the EB in that there appeared to be a few birds with less developed follicles and some morphological abnormalities. This observation was in agreement with prior research, which had indicated that embryonic EB exposure results in oviductal abnormalities and impaired reproduction [33]. These data also parallel some impacts of estrogens in mammals, which involve reproductive tract abnormalities associated with embryonic steroid exposure [13,30]. Embryonic EB treatment in male embryos resulted in sharply reduced courtship and mating behavior, as well as increased lag time to initiate behavior (26 s versus 148 s; control versus EB). Again the experimental paradigm used a relatively low dose of EB treatment compared to previous studies in an attempt to mimic the consequences of more weakly estrogenic EDCs [2,3,7,28,33,38]. As such, it is not surprising that male sexual behavior was compromised but not eliminated. Moreover, males paired with females of the same treatment had greater opportunity to copulate than males in a testing paradigm. Therefore, even behaviorally impaired males could achieve copulation in a paired, caged environment.

Subsequent studies concentrated on the characterization of EDC effects in Japanese quail. Because many of the EDCs have estrogenic action and the male Japanese quail is sensitive to the impact of estradiol, especially during

embryonic development, it is important to understand the biological effects of these compounds. The effects of EDCs are further complicated by the relatively weak endocrine activity that many of these EDCs exert.

3.2. Long-term consequences of methoxychlor exposure

3.2.1. Maternal transfer of methoxychlor from hen to egg

A number of studies have attempted to assess the transfer of chemicals from maternal systems to their offspring. In birds, the egg is an active destination for contaminants, especially the egg yolk, which sequesters lipophilic compounds. Therefore, it is important to investigate the effects of embryonic exposure to known concentrations of EDCs during embryonic development. Moreover, it has long been established that lipophilic compounds do transfer to the yolk. However, each EDC will have different relative transfer properties due to the chemistry of the compound. This is an important point when considering the field relevance of EDCs for wildlife, in that the exposure in the field is often inconsistent and likely to vary overtime, thereby affecting the availability of the compound for deposition into the yolk. As such, it is important to ascertain transfer of selected compounds to understand how these phenomena may take place in wild birds. In our study, methoxychlor (MXC; 2.5 mg) was weighed into gelatin capsules and administered orally, once daily for five consecutive days. This is clearly a high dose of MXC that we used in order to be able to chemically assess daily changes in the MXC transferred to the yolks. Eggs were collected 3 days prior to treatment and for the subsequent 13 days. Eggs collected from birds prior to MXC treatment served as control data. Eggs collected from these hens showed that MXC is maternally deposited.

Because previous data suggested that methoxychlor was not maternally transferred to eggs [37], we took advantage of improved detection methods to increase sensitivity and analyzed these samples using gas chromatography [40]. A Hewlett-Packard Model 5890 Series II gas chromatograph, was equipped as follows: 63Ni electron capture detector; packed column inlet with glass sleeve using direct injection-port mode; 30 m, 0.53 mm i.d. DB-1 wide bore capillary column (J&W Scientific, Folsom, CA); carrier gas UHP helium, 20 mL/min; make up gas argon/methane (95 + 5), 10 mL/min. Injector temperature was 250 °C, detector temperature was 250 °C, and column temperature was 230 °C [19,34].

Methoxychlor reference standards were obtained from the U.S. Environmental Protection Agency (Laurel, MD). A stock standard solution (53.52 ng/μL) was prepared in acetone. Working standard solutions, ranging from 4.1 to 1070 pg/μL were prepared in 2,2,5-trimethyl pentane (isooctane) from the stock standard solution.

The analytical procedure for methoxychlor analysis in egg yolks was as follows. Eggs were collected daily, yolk separated from albumen and stored at −20 °C until assay using a previously published method [34]. Briefly, egg yolk (1.8–2.5 g) was extracted with acetonitrile, water was added to the extract, and then was subjected to a C18 solid phase extraction cartridge cleanup, with further cleanup using a mini-Florisil column cleanup as described elsewhere [11,19]. The sample extract (3.0 μL) and a working standard of suitable concentration (3.0 μL) were injected into the gas chromatograph; concentrations of unknowns were calculated against the working standards.

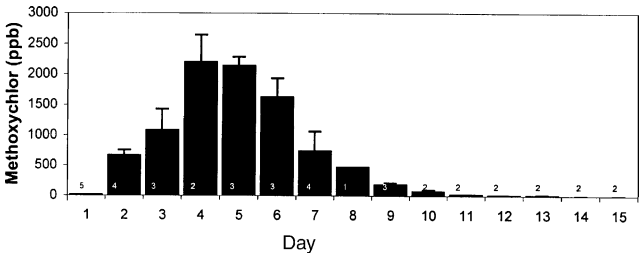

Fig. 1. Maternal transfer of MXC into egg yolk of hens given daily MXC in gel capsule; concentrations were significantly ($p < 0.05$) increased after 2 days of treatment and diminished to low levels by 4 days post-treatment.

Results showed that methoxychlor was detectable in the egg yolk two days after initiation of dosing and remained measurable 4 days after last treatment ($p < 0.05$; Fig. 1). Yolk-MXC concentrations rapidly accumulated in the yolk over the days in which hens received the bolus MXC and subsequently gradually decreased with cessation of the treatment. Day 2 methoxychlor concentrations differed significantly ($p < 0.05$) from day 1 and peaked on day 4. This pattern in the yolk MXC concentrations was associated with the follicular hierarchy in the ovary; those follicles nearing ovulation already had most their complement of yolk, whereas the smaller follicles had a longer time to collect MXC from the elevated plasma levels. Therefore, large follicles accrued limited concentrations of MXC and follicles that were lower in the follicular hierarchy accumulated relatively more MXC because these follicles were rapidly collecting yolk during the time of MXC exposure. Conversely, the gradual decline in yolk MXC content reflects both the disappearance of circulating MXC and the accumulated MXC in the yolk at the time of cessation of treatment. In total, these data provide evidence for the sequestering of lipophilic compounds in the avian egg yolk, which in turn becomes available to the chick as it develops and utilizes the yolk as a source of nutrition. In addition, the chick's blood supply to the yolk makes the yolk constituents available on a consistent basis, thereby exposing the developing chick to the compounds within the yolk. Consequently, exposure of the avian female to lipophilic EDCs, even for a brief period of time during egg formation, could result in the deposition of an EDC into the yolk, which in turn would impact the developing embryo. Again, it should be emphasized that the dose of MXC (2.5 mg/day) is very high and much greater than would be associated with field exposure. Because this pesticide has a wide working range and is relatively non lethal, it was possible for us to use this dose to assess maternal transfer. Therefore, our subsequent studies

utilized much lower doses, both for egg injection and for dietary exposure studies. These studies will be presented below.

3.2.2. Effects of methoxychlor egg injections on neuroendocrine systems and sexual behavior

Our initial studies with MXC were egg injection studies because we wished to characterize the impact of known exposures at selected times in embryonic development. A range of doses was used to ascertain effects on neuroendocrine and behavioral end-points. A single injection was administered, and it is assumed that the entire dose is gradually absorbed during embryonic development. Further, because there are enzymes present in the egg, some of the active metabolites of MXC would also be produced and mimic field exposures to avian embryos. Fertilized Japanese quail eggs were injected with 0, 150 or 300 μg MXC/egg at E4 [28,30]. Early embryonic exposure did not impact survival of embryos at these relatively low levels. In addition, long-term effects were observed in the behavioral responses in males. However, there was a great deal of variability across males in their behavioral responses, especially over the three consecutive days of behavioral testing. This suggests that MXC may affect behavior and there may be some changes in behavioral responses with experience. The current study was not designed to examine this issue; however, it would be of interest to follow up on this study and address potential interactions between experience and EDC exposure. Similarly, no consistent effects were observed in the plasma steroid hormones in adult males and females that had been exposed to MXC. Again, there was variability between individuals in plasma hormone levels, suggesting diverse response of individuals to embryonic EDC exposure. Measurements of hypothalamic GnRH-I concentrations showed effects of embryonic MXC exposure, especially in hatchlings rather than in adults. Interestingly, many of these differences disappeared when measured in adults. We observed a similar perinatal alteration with embryonic exposure to vinclozolin [18]. These observations provide support for the hypothesis that EDC effects on the HPG axis may be more evident at hatch and compensatory mechanisms are manifest as the animal matures. Similar observations have been made in both the field and laboratory in which ovotestes are observed at hatch, but rarely in persist adult birds (for review, see [30]). Hence, it is important to assess the impact of EDCs at several phases in the life cycle and to be attentive to individual variation in response as the variability may be an excellent indicator of impact that could be significant at a population level.

3.3. Long-term consequences of dietary methoxychlor

3.3.1. Maternal transfer of dietary methoxychlor from hen to egg

A feeding study was conducted to assess transfer of methoxychlor at environmentally relevant levels. Methoxychlor was mixed into feed at three doses, which were field relevant: control (0 ppm, $n = 3$), low (0.5 ppm, $n = 5$), high (5.0 ppm, $n = 5$). Eggs were collected after 8 weeks of treatment and stored at 4 °C for up to 1 week. Yolks were separated from albumens, combined into two pools per treatment and frozen at −80 °C until analysis. Yolk MXC was determined according to the protocol described above.

A subset of eggs collected from birds fed dietary MXC were analyzed for MXC concentrations. The highest dietary MXC concentration (5 ppm) was associated with detectable levels of MXC (5+ ppb) in the yolk of 50% of the eggs, with 33% of the eggs having levels greater than 10 ppb MXC. In the low dietary MXC (0.5 ppm) hens, 50% of the eggs had detectable MXC concentrations; however, these concentrations of MXC were between 2 and 6 ppb. This supports the hypothesis that the yolk serves as a potential depot for lipophilic EDCs and even when the dietary levels of an EDC are at relatively low, field relevant levels. Furthermore, by its presence throughout embryonic development, an EDC is likely to impact a number of endocrine-associated processes, including sexual differentiation.

3.3.2. Effects of dietary methoxychlor on neuroendocrine systems and sexual behavior

Multigenerational studies provide a format for study of individuals exposed via diet and maternal deposition, as well as through significant portions of their life span. In these studies, Japanese quail breeders were exposed to low levels of dietary MXC (0, 0.5 and 5 ppm), with continued exposure in their offspring (F1), and no treatment of the F2 chicks. This test design allowed assessment of reproductive endocrine and behavioral end-points in birds exposed to dietary MXC at a number of phases in the life cycle. Many of the traditional toxicological end points measured including fertility, hatching success, and 14-day viability did not show discernable effects of the dietary MXC. Both F1 and F2 male offspring exposed to MXC showed impaired mating behavior. More detailed description of this study and the design follow.

Proven breeding pairs (P1) were chosen and randomly assigned into three groups of dietary MXC: control (0 ppm), low (0.5 ppm), and high (5.0 ppm). After 4 weeks of treated diet, eggs were collected and incubated to produce the second generation (F1). The P1 birds were sampled after 8 weeks of adult exposure to dietary MXC. These animals experienced lifetime exposure to MXC, both in ovo (via maternal deposition) and after hatch. After reaching adulthood, a third generation (F2) was incubated and raised on control feed. These animals had only in ovo exposure to MXC and thus any changes would be attributable to maternal environment. General reproductive parameters such as egg productivity, fertility, hatching success and 14-day viability were recorded. There were 20 pairs/dietary treatment. At sexual maturity, F1 and F2 males were tested for sexual behavior ($n = 15$/dietary treatment group). Naïve males were singly housed and a reproductive female was introduced into the male's home cage. Latency to mount, number of mount attempts and number of successful cloacal contacts were recorded for 3 min on three consecutive days.

Results showed that MXC, even at low dietary levels had some effects in the P1 pairs, with reduced plasma estradiol in females and lower circulating androgen in treated males. However, no differences were observed in other reproductive responses, including fertility, egg production, hatching success or viability of chicks. In the F1 and F2 birds, traditional toxicological end-points, including fertility, hatching success, and 14-day viability did not show evidence of MXC impact. However, there were discernable effects of dietary MXC on reproductive endocrine, neuroendocrine, and behavioral end-points. MXC exposure affected male sexual behavior over the 3 days of testing (Figs. 2 and 3), relative to latency to mount, number of mount attempts, and number of successful mount attempts. In both the F1 and F2 males, experience over the 3 days of testing failed to improve performance in the high MXC males. Although there was no statistical difference in latency to mount, it may be seen in Fig. 2a that the high MXC males showed little change in their latency to mount, whereas males in the control and low MXC groups improved over the 3-day trial. In the F1 males, high MXC males had fewer successful cloacal contacts; this behavioral difference persisted on the 3 days of behavioral testing, especially in the number of completed matings (Fig. 2b and c). There is also variability in these behavioral responses. In our experience with working with the EDCs, there is generally a bimodal response, with some individuals showing more sensitivity to EDC impact. As such, variability becomes an important end-point and should be considered as one of the measurement

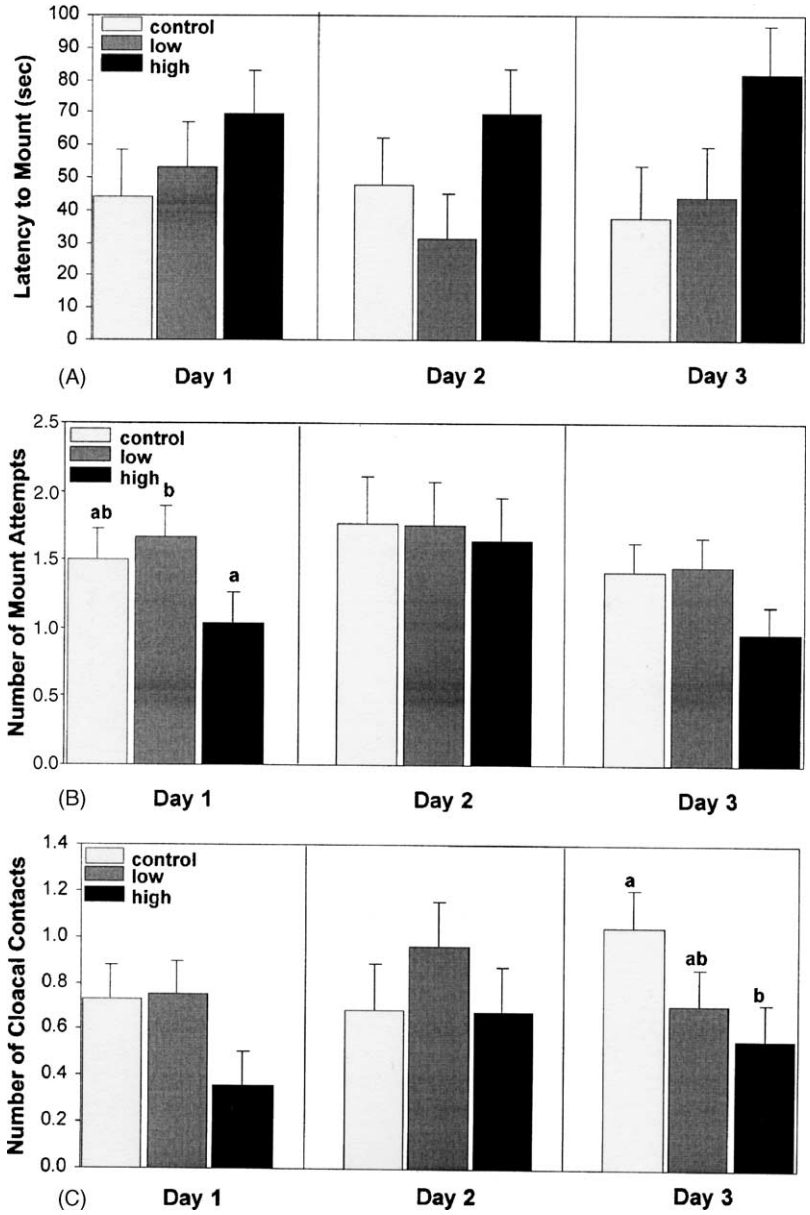

Fig. 2. Reproductive behavior in F1 males ($n = 15$/dietary treatment group) exposed to 0, 0.5 or 5 ppm MXC in their diet in a two-generation study; different letters denote significant differences ($p < 0.05$) between pairs.

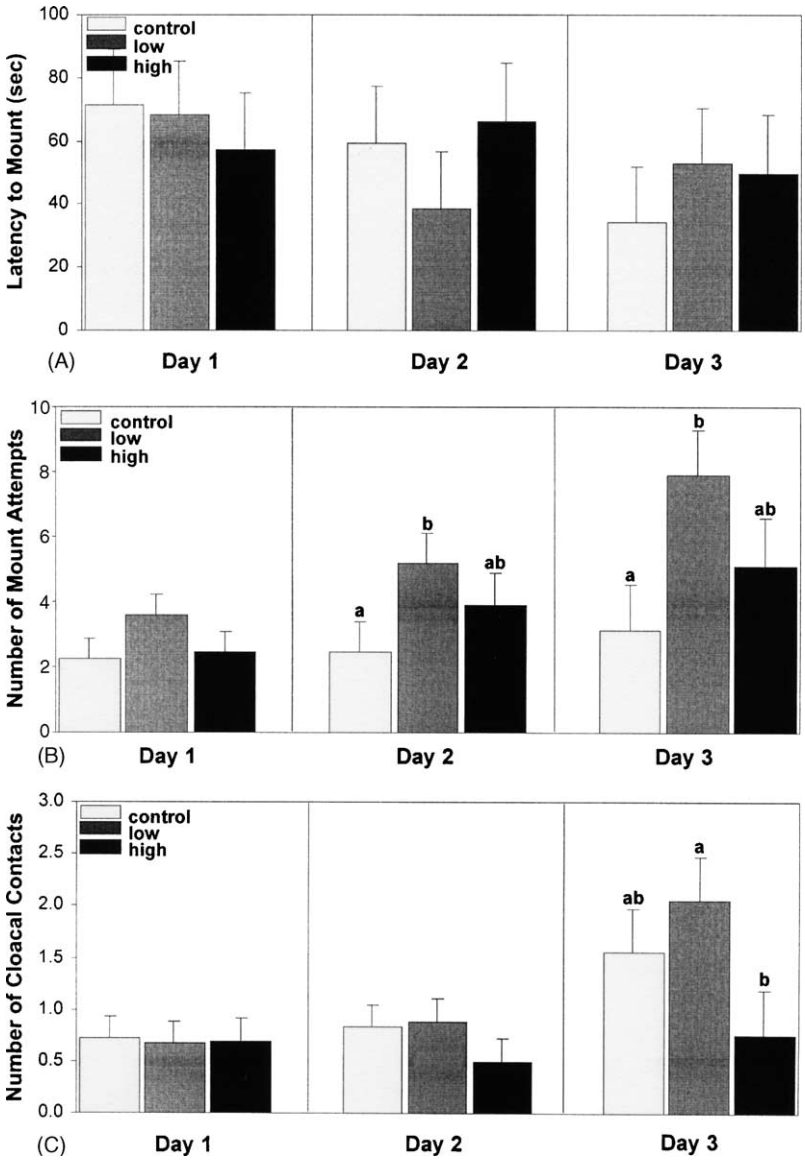

Fig. 3. Reproductive behavior in F2 males ($n = 15$/dietary treatment group) exposed to 0, 0.5 or 5 ppm dietary MXC in a two-generation study; different letters denote significant differences ($p < 0.05$).

end points for EDC exposure. Finally, when the results were averaged over the three days of testing, the data were consistent with the analysis for the separate days. These data suggest that experience does not enhance the performance of the individual that has been embryonically exposed to MXC. In contrast, in the F2 males, the low treatment males performed more mount attempts as well as number of successful cloacal contacts, compared to the other two groups (Fig. 3a, b and c), although these differences were not always significant. These results are more variable than the behavioral responses in the F1 males, suggesting that the combination of embryonic MXC exposure and dietary MXC in the F1 males had quantitatively more effect than embryonic exposure alone in the F2 males. Again, variability in response may be associated with individual sensitivities to EDC exposure and as such may be an important measurement end-point.

Hypothalamic catecholamines were assayed at selected ages to examine the neurotransmitters: EPI, NE, DA and 5-HT. The P1 birds were analyzed after 8 weeks of adult exposure to dietary MXC (Fig. 4a and b). Among females, NE levels were elevated ($p < 0.5$) in the high group, while DA was depressed in the low group. Male NE and DA followed a similar pattern, however, EPI levels were reduced as well. F1 chicks were sampled on the day of hatch (Figs. 5 and 6). These F1 chicks were hatched from fertile eggs collected from P1 pairs during either experimental week 3 of dietary MXC (F1-3) or experimental week 6 of dietary MXC (F1-6). This comparison was conducted to see if there was an accumulative effect of dietary MXC. Results showed that NE levels were reduced in the high F1-3 females, while no differences were detected in the F1-3 males (Fig. 5). F1-6 females had reduced EPI in the high MXC group, while

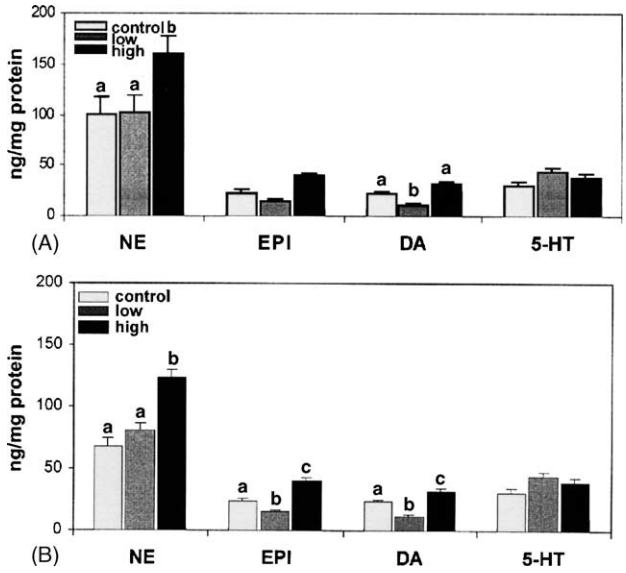

Fig. 4. Hypothalamic monoamines (NE = norepinephrine, E = epinephrine, DA = dopamine, 5HT = serotonin) in P1 birds (n = 8/sex/dietary treatment group) in the two-generation study; with 0, 0.5 and 5 ppm dietary MXC; different letters denote significant differences.

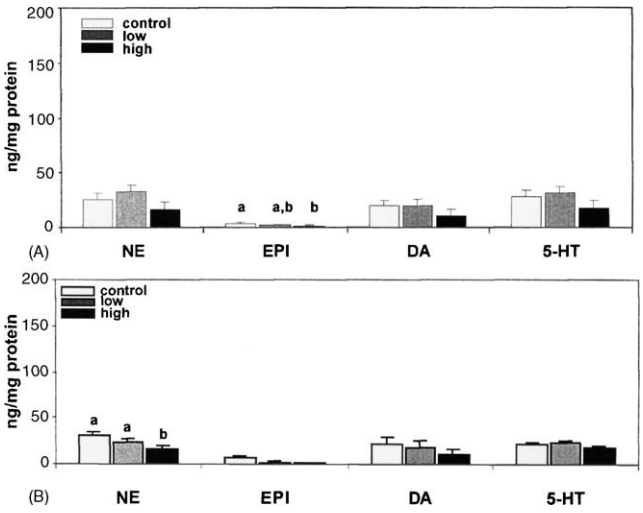

Fig. 6. Hypothalamic monoamines (NE = norepinephrine, EPI = epinephrine, DA = dopamine, 5HT = serotonin) in F1 birds (n = 8/sex/dietary treatment group) that were sampled at day 1, post-hatch. Birds were hatched from eggs collected from P1 pairs at week 6 of dietary MXC treatment in the two-generation study; with 0, 0.5 and 5 ppm dietary MXC; different letters denote significant differences.

F1-6 males had reduced NE with high MXC (Fig. 6). F1 birds were also sampled after reaching adulthood (F1-AD), but catecholamines were not different between groups (data not shown). Catecholamines were also determined in F2 birds, at hatch and as adults. No significant differences were detected in the F2 birds; however, NE and EPI levels showed similar trends in the males with lower concentrations in the high MXC adult males. These differences observed in

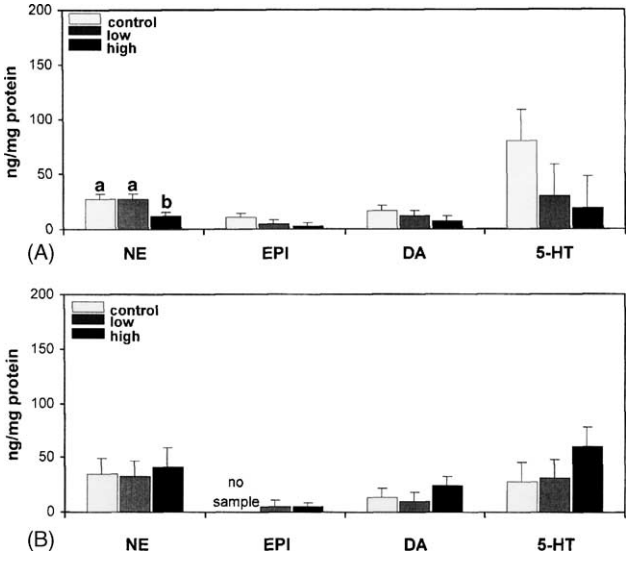

Fig. 5. Hypothalamic monoamines (NE = norepinephrine, EPI = epinephrine, DA = dopamine, 5HT = serotonin) in F1 chicks (n = 8/sex/dietary treatment group) sampled at day of hatch. These chicks were hatched from eggs collected from the PI pairs during week 3 of MXC treatment. Birds were part of a two-generation study; with 0, 0.5 and 5 ppm dietary MXC; different letters denote significant differences.

catecholamines are important because these neurotransmitters stimulate GnRH-I release [30]. Further, hypothalamic NE and EPI concentrations increase at the time of sexual maturation, probably in association with increasing activity of the GnRH-I system and rising levels of gonadotropins essential for initiation of gonadal function. In addition, hypothalamic NE and EPI are also important in modulating reproductive behavior; reduced NE and/or EPI are likely to be associated with diminished male sexual behavior, such as the decreased behavior observed in the F1 males. Although the decrease in NE and EPI were not significant in F2 males, this trend is similar to that observed in the F1 males; these observed differences in neurotransmitters may underly the observed diminished behavior. These data point toward the hypothalamic catecholamines as important indices of EDC exposure, especially in the case of estrogenic EDCs and their impact on reproductive behavior and neuroendocrine systems in the male. Further studies are needed to assess the consequences of these differences in concentration, including turnover assessment and measurement of appropriate receptors. If these differences in neurotransmitter contents are indicative of functional differences, it would be of interest to conduct some type of endocrine challenge to determine if the HPG axis is impaired. Finally, it is important to determine if an EDC, such as embryonic MXC exposure, has reproductive consequences observable in a dose-related manner. If this is the case, then behavioral and neurochemicals have potential utility as indices which are predictive of reproductive impact in a graded, or dose-related manner.

These data are interesting for several reasons. First, dietary MXC had some impact on the HPG axis, but it was not sufficient to alter reproductive performance, suggesting that a threshold of “impact” is necessary in order to result in

Table 1
Potential measurement end-points for assessing EDCs in birds (adapted from the OECD Expert Group on Assessment of EDC Effects in Birds Report, 2001, and references [2,7,12,20,25,31,32,36])

Category of endpoint	Timing of exposure	Duration of EDC effects	Detectable in hatchling?	Detectable effect in adults?
Fitness	Embryonic (exposure via maternal deposition)	Long-term		
Hatching			Yes	–
Growth			Yes	–
Fertility			Yes	Yes
Gonad morphology			Yes	Yes
Behavioral	Embryonic	Long-term		
Separation/open field behavior			Yes	–
Sexual behavior	Adult (exposure via diet or transdermal)	Short- and long-term	–	Yes
Parental behavior			–	Yes
Endocrine Axis	Embryonic	Long-term		
Sexual maturation			–	Yes
Peak reproduction	Adult	Short- and long-term	–	Yes
Reproductive failure			–	Yes
Aging			–	Yes
Neuroendocrine and hormones	Embryonic	Long-term		
Catecholamines	Adult		Yes	Yes
Indolamines		Short- and long-term	Yes	Yes
Aromatase enzyme			Yes	Yes
GnRH-I			Yes	Yes
Vasotocin			?	Yes
Plasma hormones			Yes	Yes

reproductive impairment. Second, the F1 birds are exposed via maternal deposition of MXC into the egg as well as in their diet. Some of the main effects noted in these birds appeared to be in a delay of sexual maturation. These data suggest that many of the traditional end-points measured in toxicological studies are not sufficiently sensitive or perhaps most appropriate to detect EDC effects.

4. Concluding remarks

The potential impact of EDCs on field populations of birds remains unclear. However, the current studies and data from other laboratories in combination with records from field exposures (often involving lethal exposures for a number of individuals) are convincing support for further characterization of these compounds. This includes both understanding their mode of action as well as investigating the consequences of early exposure on reproductive success and lifetime reproductive performance. Furthermore, it is important to develop a panel of measurement end-points for assessment of potential EDCs, which is critical for evaluating EDC effects in field birds. These measurement end-points also must consider varied sensitivity to endocrine disruption with stage of life. Ideally, in order for the measurement end-points to be useful for ecological risk assessment in field birds, it is important that there be a dose-dependent response to the EDC. Although exposure is often non lethal, especially in the field, the subtle life long impact of early exposure is likely to have transgenerational effects and possibly slowly erode population vigor and viability. An overview of categories of these potential measurement end points is shown in Table 1 (from [25]). As may be seen on Table 1, measures often considered as indicative of fitness do show long-term effects of embryonic EDC exposure. However, our data and observations from other laboratories suggest that these measures of fitness may not be sufficiently sensitive to the impact of some EDCs. As such, these measures are important, especially for more potent EDCs. Conversely, neuroendocrine and behavioral measures appear to be sensitive and reliable indicators of embryonic EDC exposure. Similarly, the timing of sexual maturation, plasma hormones, and reproductive function of adults during the period of peak productivity often follow the responses of the neuroendocrine systems that modulate the reproductive axis. Although few data have been collected on reproductive failure and aging in birds exposed to EDCs, this is an important issue that deserves research as we learn more about the action of EDCs.

At this time, the studies used to develop the summary provided in Table 1 provide verification of the efficacy of many of these measurement end-points as reliable indices of EDC exposure. Therefore, use of these measures becomes particularly relevant for consideration in assessing the long-term impact of EDCs on birds because they are sexually dimorphic and organized under the influence of steroid hormones during embryonic development. Finally, these studies support the use of the Japanese quail embryo as a useful model for early EDC exposure and for assessing the consequences of EDCs on neuroendocrine and behavioral responses on the maturing and adult individual.

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References

[1] M.A. Abdelnabi, M.R. Bakst, J.E. Woods, M.A. Ottinger, Plasma 17-β estradiol levels and ovarian interstitial cell structure in embryonic Japanese quail, Poult. Sci. 79 (2000) 564–567.
[2] M.A. Abdelnabi, M.A. Ottinger, Hypothalamic indolamines during embryonic development and effects of steroid exposure, Gen. Comp. Endocrinol. 130 (2003) 13–19.
[3] E. Adkins-Regan, Hormonal basis of sexual differentiation in birds, in: J. Balthazart (Ed.), Hormones, Brain and Behavior in Vertebrates. 1. Sexual Differentiation, Neuroanatomical Aspects, Neurotransmitters and Neuropeptides, vol. 8, Comp. Physiol. Basel., Karger, 1990, pp. 1–14.
[4] E.K. Adkins-Regan, M.A. Ottinger, J. Park, Maternal transfer of estradiol to egg yolks alters sexual differentiation of avian offspring, J. Exp. Zool. 271 (1995) 466–470.
[5] L.C. Alworth, K.L. Howdeshell, R.L. Ruhlen, J.K. Day, D.B. Lubahn, T.H.-M. Huang, C.L. Besch-Williford, F.S. vom Saal, Uterine responsiveness to estradiol and DNA methylation are altered by fetal exposure to diethylstilbestrol and methoxychlor in DC-1 mice: effects of low versus high doses, Toxicol. Appl. Pharmacol. 183 (2002) 10–22.
[6] S.Y. Amstislavsky, E.A. Kizilova, V.P. Eroschenko, Preimplantation mouse embryo development as a target of the pesticide methoxycholor, Reprod. Toxicol. 17 (2003) 79–86.
[7] J. Balthazart, E. Adkins-Regan, in: D. Pfaff, A. Arnold, A. Etgen, R. Rubin (Eds.), Sexual differentiation of brain and behavior in birds, 4, Academic Press, 2002, pp. 223–302.
[8] J. Balthazart, M.A. Ottinger, 5β-reductase activity in the brain and cloacal gland of male and female embryos in the Japanese quail *(Coturnix coturnix japonica*), J. Endocrinol. 102 (1984) 77–81.
[9] C. Berg, K. Halldin, A.K. Fridolfson, I. Brandt, B. Brunstrom, The avian egg as a test system for endocrine disruptors: effects of diethylstilbestrol and ethynylestradiol on sex organ development, Sci. Total Environ. 233 (1999) 57–66.
[10] J.L. Carlson, M.R. Bakst, M.A. Ottinger, Developmental stages of primary oocytes in turkeys, Poult. Sci. 75 (1996) 1569–1578.
[11] D.R. Erney, A feasibility study of miniature florisil columns for the separation of some chlorinated pesticides, Bull. Environ. Contam. Toxicol. 12 (1974) 717.
[12] A. Elbrecht, R.G. Smith, Aromatase enzyme activity and sex determination in chickens, Science 255 (1992) 467–468.
[13] V.P. Eroscenko, S.Y. Amstislavsky, H. Schwabel, R.L. Ingermann, Altered behaviors in male mice, male quail, and salamander larvae following early exposures to the estrogenic pesticide methoxychlor, Neurotoxicol. Teratol. 24 (2002) 29–36.
[14] N.H. Golden, B.A. Rattner, P.C. McGowan, K.C. Parsons, M.A. Ottinger, Concentrations of metals in feathers and blood of nestling black-crowned night-herons (*Nycticorax nycticorax*) in Chesapeake and Delaware Bays, Bull. Environ. Contam. Toxicol. 70 (2003) 385–393.
[15] N.H. Golden, B.A. Rattner, J.B. Cohen, D.J. Hoffman, E. Russek-Cohen, M.A. Ottinger, Lead accumulation in feathers of nestling black-crowned night herons (*Nycticorax nycticorax*) experimentally treated in the field, Environ. Toxicol. Chem. 22 (2003).
[16] K. Halldin, C. Berg, I. Brandt, B. Brunstrom, Sexual behavior in Japanese quail as a test end point for endocrine disruption: effects of in ovo exposure to ethinylestradiol and diethylstilbestrol, Environ. Health Perspect. 107 (1999) 861–866.
[17] C. Latchoumycandane, P.P. Mathur, Induction of oxidative stress in the rat testis after short-term exposure to the organochlorine pesticide methoxychlor, Arch. Toxicol. 76 (2002) 692–698.
[18] Q. Li, G.F. Paciotti, L. Tamarkin, M.A. Ottinger, LHRH-I release from quail hypothalamic slices measured by specific EIA, Gen. Comp. Endocrinol. 95 (1994) 13–24.
[19] Q. Li, L. Tamarkin, P. Levantine, M.A. Ottinger, Estradiol and androgen modulate chicken LHRH-I release in vitro, Biol. Reprod. 51 (1994) 896–903.
[20] S. McGary, P.F.P. Henry, M.A. Ottinger, Impact of vinclozolin exposure on Japanese quail reproduction, Environ. Toxicol. Chem. 20 (2002) 2487–2493.
[21] B.M. McMahon, R. Wagner (Eds.) Pesticide Analytical Manual, Volume I, Section 304, section C-7 (1994) U.S. Food and Drug Administration, Washington, DC.
[22] J.R. Millam, C.B. Craig-Veit, M.E. Batchelder, M.R. Viant, T.M. Herbeck, L.W. Woods, An avian bioassay for environmental estrogens: the growth of zebra finch (*Taeniopygia guttata*) chick oviduct to oral estrogens, Environ. Toxicol. Chem. 21 (2003) 2663–2668.
[23] M.A. Ottinger, Sexual differentiation of neuroendocrine systems and behavior, Poult. Sci. 68 (1989) 979–989.
[24] M.A. Ottinger, A.M. Abdelnabi, Neuroendocrine systems and avian sexual differentiation, Am. Zool. 37 (1997) 514–523.
[25] M.A. Ottinger, M.A. Abdelnabi, M. Quinn, N. Golden, J. Wu, N. Thompson, Reproductive consequences of EDCs in birds: What do laboratory effects mean in field species? Neurotoxicol. Teratol. 24 (2002) 17–29.
[26] M.A. Ottinger, M.R. Bakst, Peripheral androgen concentrations and testicular morphology in embryonic and young male Japanese quail, Gen. Comp. Endocrinol. 43 (1981) 170–177.
[27] M.A. Ottinger, H.J. Brinkley, Testosterone and sex-related behavior and morphology: Relationship during maturation and in the adult Japanese quail, Horm. Behav. 11 (1978) 175–182.
[28] M.A. Ottinger, H.J. Brinkley, The ontogeny of crowing and copulatory behavior in male Japanese quail *(Coturnix coturnix japonica*), Behav. Proc. 4 (1979) 43–47.
[29] M.A. Ottinger, H.J. Brinkley, Testosterone and sex-related physical characteristics during the maturation of the male Japanese quail (*Coturnix coturnix japonica*), Biol. Reprod. 20 (1979) 905–909.
[30] M.A. Ottinger, M.A. Abdelnabi, P.F.P. Henry, S. McGary, N. Thompson, J. Wu, Neuroendocrine and behavioral implications of endocrine disrupting chemicals in quail, Horm. Behav. 40 (2001) 234–247.
[31] M.A. Ottinger, I.C.T. Nisbet, C.E. Finch, Aging and reproduction: Comparative endocrinology of the common tern and Japanese quail, Am. Zool. 35 (1995) 299–306.
[32] M.A. Ottinger, S. Pitts, M.A. Abdelnabi, Steroid hormones during embryonic development in Japanese quail: plasma, gonadal, and adrenal levels, Poult. Sci. 80 (2001) 795–799.
[33] M.A. Ottinger, F.S. vom Saal, in: D. Pfaff, A. Arnold, A. Etgen, R. Rubin (Eds.), Impact of Environmental Endocrine Disruptors on Sexual Differentiation in Birds and Mammals, 4, Academic Press, 2002, pp. 325–383.
[34] G.C. Panzica, N. Aste, C. Viglietti-Panzica, M.A. Ottinger, Structural sex differences in the brain: influence of gonadal steroids and behavioral correlates, J. Endocrinol. Invest. 18 (1995) 232–252.
[35] F.M.R. Perrin, S. Stacey, A.M.C. Burgess, U. Mittwoch, A quantitative investigation of gonadal feminization by diethylstilbestrol of genetically male embryos of the quail *Coturnix japonica*, J. Reprod. Fertil. 103 (1995) 223–226.
[36] M. Quinn, J.B. French, A.F. McNabb, M.A. Ottinger, The effects of polychlorinated biphenyls (Aroclor 1242) on thyroxine, estradiol, molt, and plumage characteristics in the American Kestral (*Falco sparverious*), Environ. Toxicol. Chem. 21 (2002) 1417–1422.

[37] B.A. Rattner, R.N. Clarke, M.A. Ottinger, Depression of plasma luteinizing hormone concentration in quail by the anticholinesterase insecticide parathion, Comp. Biochem. Physiol. 83C (1986) 451–453.
[38] B.A. Rattner, M.A. Ottinger, Contaminant exposure and effects—terrestrial vertebrates database: trends and data-gaps for Atlantic coast estuaries. Environmental Monitoring and Assessment, Environ. Monitor. Assess. 63 (2000) 131–142.
[39] E.F. Rissman, M. Ascenzi, P. Johnson, E. Adkins-Regan, Effect of embyronic treatment with oestradiol benzoate on reproductive morphology, ovulation and oviposition and plasma LH concentrations in female quail (*Coturnix coturnix japonica)*, J. Reprod. Fertil. 71 (1984) 411.
[40] F.J. Schenck, R. Wagner, M.K. Hennessy, J.L. Okrasinski, Screening procedure for organochlorine and organophosphorus pesticide residues in eggs using tandem solid phase extraction cleanup, J. Assoc. Off. Anal. Chem. 77 (1994) 1036.
[41] Y. Takeuchi, T. Kosaka, K. Hayashi, M. Takeda, Y.T. Yoshida, H. Fujisawa, S. Teramoto, K. Maita, T. Harada, Thymic atrophy induced by meethoxychlor in rat pups, Toxicol. Lett. 135 (2002) 199–207.
[42] Y. Tanabe, T. Nakamura, K. Fujioka, O. Doi, Production and secretion of sex steroid hormones by the testes, the ovary, and the adrenal glands of embryonic and young chickens, Gen. Comp. Endocrinol. 39 (1979) 26–33.
[43] A.C. Waldron, E.C. Naber, Importance of feed as an unavoidable source of pesticide contamination in poultry meat and eggs, Poult. Sci. 53 (1974) 1428–1435.
[44] J.M. Whitsett, E.W. W Irvin, F.W. Edens, J.P. Thaxton, Demasculinization of male Japanese quail by prenatal estrogen treatment, Horm. Behav. 8 (1977) 254–263.
[45] L. Wittingham, H. Schwabl, Maternal testosterone in tree swallow eggs varies with female aggression, Anim. Behav. 63 (2002) 63–76.