Sexual Behavior in Japanese Quail as a Test End Point for Endocrine Disruption: Effects of *in Ovo* Exposure to Ethinylestradiol and Diethylstilbestrol

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Chemicals having a capacity to disturb the endocrine system have attracted considerable interest during recent years. There is a shortage of well-characterized *in vivo* tests with which to study such disturbances in different classes of vertebrates. In the present study, test end points related to reproduction in the Japanese quail were used to examine the estrogenic activity of chemicals. The synthetic estrogens ethinylestradiol (EE_2) and diethylstilbestrol (DES), used as model compounds, were injected into the yolk of embryonated eggs. After the birds had been raised to sexual maturity, we examined sexual behavior, plasma testosterone concentrations, and testis morphology in adult males. The lowest doses resulting in a significantly depressed male sexual behavior were 6 ng/g egg for EE_2 and 19 ng/g egg for DES. Testis weight asymmetry was increased at 6 ng EE_2/g egg, but DES had no effect at any treatment level. The area of the androgen-dependent cloacal gland was significantly reduced at 57 ng DES/g egg. No effects on plasma testosterone concentration or body weight following exposure to EE_2 or DES were observed at any dose level. Depressed male sexual behavior was the most sensitive of the end points studied, and we suggest that this ecologically relevant end point be included in avian *in vivo* testing for neuroendocrine disruptors. *Key words:* diethylstilbestrol, endocrine disruption, environmental estrogens, ethinylestradiol, Japanese quail, sexual behavior, testis, testosterone, test system. *Environ Health Perspect* 107:861–866 (1999). [Online 1 October 1999]
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The hypothesis that environmental contaminants can disrupt endocrine function in wildlife and in humans has attracted much attention during recent years. Numerous experimental studies have shown that reproduction may be at risk from chemicals discharged into the environment (*1–8*). Moreover, studies in wild species belonging to various animal classes have suggested that reproductive impairment may be caused by environmental contaminants (*9–12*). Reproductive effects are often insidious and difficult to detect, and sometimes pass undetected until population density is severely reduced.

Laboratory studies have shown that some environmental contaminants act as estrogen receptor agonists, and such chemicals are suspected to have caused estrogen receptor-mediated disorders in wildlife. For example, feminization of male gull embryos occurred following injections of DDT at concentrations similar to those found in gull eggs from southern California (*13*). On the basis of these findings, Fry et al. (*14*) postulated that depression of breeding behavior in male gulls by DDT compounds could be the cause of reported female–female pairing and sex ratio skew in colonies of breeding gulls.

Numerous assays for the identification of endocrine disruptors have been described. Most are *in vitro* tests based on hormone receptor binding or hormone-dependent cell proliferation (*15–19*). The risk of producing false negatives in these assays, mainly due to lack of metabolic capacity, is of considerable concern. Cells having some inherent metabolic activity (*20*) and media containing hormone-binding proteins (*21*) are used to imitate the *in vivo* situation. However, when using data obtained from *in vitro* testing, it is still not possible to predict effects on the whole organism, which is why *in vivo* tests for detection of substances that disturb endocrine function are required. *In vivo* tests take into consideration many of the problems associated with interpretation of results from *in vitro* assays. It is possible to account for metabolism, distribution, target organ availability, and excretion of various compounds in *in vivo* tests. Furthermore, effects of ecologic relevance can be studied. Mammalian *in vivo* tests for endocrine-mediated effects are currently used in laboratories worldwide. Examples of *in vivo* tests for estrogenic activity in rodents are the uterotrophic test (*22,23*) and the prostate weight test (*21,24*). Avian tests are not as developed or as widely used as mammalian tests. A consequence of the lack of standardized tests for endocrine disruption in avian species is that much knowledge of avian endocrinology has not yet been incorporated into the field of toxicology and environmental science.

In mammals, the female is considered to be the "nonhormonal" sex, meaning that sexually dimorphic structures develop in a feminine manner in the absence of sex hormones. In birds, however, the male is regarded as the nonhormonal sex and estrogen plays a key role in differentiation of sexual dimorphism. The role of estrogens in avian sexual differentiation is primarily to inhibit development of masculine characteristics (*25*). In the Japanese quail, sexual dimorphism in terms of behavior and neuroendocrinology has been extensively studied for over 25 years [see Balthazart et al. (*26*) for review]. The importance of estrogen for the organization of behavioral, neurochemical, and neuroanatomical features is well established (*25,27–31*). Female Japanese quail are demasculinized by endogenous estrogens and fail to show male-typical sexual behavior in response to testosterone treatment in adult life (*32,33*). Likewise, embryonic treatment with estrogen during a critical period of brain development results in an irreversibly depressed response in the adult male to the activating effects of testosterone on copulatory behavior (*27,28*); that is, exogenous estrogens organize the male brain in a nonmasculine way during embryogenesis. The critical period for this demasculinization ends around day 12 of incubation (*28,29*).

Sexual behavior in male Japanese quail consists of well-defined sequences (*34,35*) that are easily observed and recorded under laboratory conditions. Male copulatory behavior consists of neck grab, mount attempt, mount, and cloacal contact movement. The sexual activity of the mature male quail is generally very vigorous, thus offering good opportunities to study deviations from normal reproductive behavior.

The objective of this study was to devise a test for detection of estrogenic effects in male Japanese quail, the ultimate objective being to examine sexual behavior in estrogen-treated birds. Behavioral aberrations have been suggested as an important mechanism of endocrine disruption in birds (*36*). We examined changes in sexual behavior in

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relation to testis morphology and plasma testosterone concentration.

The synthetic estrogens ethinylestradiol (EE_2) and diethylstilbestrol (DES) were the test compounds used in this investigation. EE_2 is used as a contraceptive and has been identified as a widespread environmental pollutant released via effluent water from sewage treatment works in the United States (*37*) and Great Britain (*38*), and recently, also in Sweden (*39*). High concentrations of conjugated EE_2 were found in bile of rainbow trout caged downstream of a Swedish sewage treatment plant (*39*). DES is a synthetic estrogen that was widely used in the 1950s and 1960s to prevent miscarriage. Its use resulted in reproductive organ dysfunction in children of exposed women. The Japanese quail was chosen as the test species because it has been widely used as a research animal and its physiology and neuroendocrinology are well characterized.

The study presented here was conducted as two different experiments in which male quail embryos were exposed to the test compounds *in ovo*. In the first experiment, dose–response relationships for EE_2 and DES were studied regarding male sexual behavior, testis weight, testis morphometry, and plasma testosterone concentration in the adult bird. In the second experiment, DES-treated prepubertal male birds had a Silastic (Noax AB, Stockholm, Sweden) tube filled with testosterone implanted to activate sexual behavior in order to determine whether the estrogen-induced depression of sexual behavior observed in the first experiment was most likely due to a persisting effect on testosterone production or to an organizational effect on the brain.

Materials and Methods

Animals and chemicals. Fertilized Japanese quail eggs weighing 11.8 ± 0.9 g [mean ± standard deviation (SD)] were obtained from a local breeder. 17α-Ethinylestradiol (purity ≥ 98%), diethylstilbestrol (purity ≥ 99%), and testosterone (purity > 99%) were purchased from Sigma Chemical Co., St. Louis, Missouri.

The eggs were incubated at 37.5°C and 60% relative humidity and turned every third hour. DES and EE_2 were dissolved in an emulsion of peanut oil, lecithin, and water (*40,41*) and injected into the yolks on day 3 of incubation. Controls received emulsion only. Eggs were injected (20 µl/egg) with a 27-gauge syringe through a small hole punched with a needle at the blunt end of the egg. Holes were sealed with melted paraffin wax and the eggs were returned to the incubator. On day 15 of incubation, the eggs were placed in hatching boxes at 37°C and 70% relative humidity until hatching occurred between day 17 and 19. After hatching, the chicks were raised in heterosexual groups with turkey starter and tap water provided *ad libitum*. The animal facility was lit 16 hr/day. One week before starting behavioral testing, the males were individually placed in metal cages with wood shavings on the floor.

Behavioral testing. Sexual behavior was studied in a test arena (50 × 40 cm; height 30 cm) with plywood walls, roof, and floor. The front of the test arena was a metal grid that allowed visual observation of the birds. The floor was covered with wood shavings. A receptive egg-laying female was placed in the test arena, and the male was introduced shortly after. Sexual interactions were observed for 2 min. The behaviors scored were neck grab, mount attempt, mount, and cloacal contact movement. A mount attempt was scored when a male, while neck grabbing, put one leg over female's back. As suggested by preliminary studies, the neck grab and mount attempt were considered less dependent on the receptivity of the female than were the mount and cloacal contact movement. A similar conclusion was drawn by Alexandre and Balthazart (*42*). The mount attempt was therefore used in the statistical analysis of the behavioral data. One test was performed each day on 5 consecutive days. Males that made at least one mount attempt within the 2-min test period were deemed sexually active. The performance of each bird was scored on a scale from 0 to 5, depending on the number of positive tests. The scores of the birds in the EE_2- and DES-treated groups were statistically compared with those of their corresponding control group. To reduce the risk of bias due to differences in receptivity of the females, each male was tested daily with a different female than the day before. All tests were performed as blind tests.

Testosterone analysis. Blood for testosterone analysis was collected from the wing vein 1–2 days after the last behavioral test. All blood samples were collected within 1 hr in the morning so that blood was not sampled close to the reported nocturnal surge of testosterone secretion (*43*). The blood samples were immediately placed on ice, centrifuged, and frozen at -20°C until analyzed. Testosterone was determined using a solid phase radioimmunoassay (RIA) kit (Coat-A-Count, Diagnostic Products Co., Los Angeles, CA) according to the manufacturer's recommendations. Serial dilutions of quail plasma containing high concentrations of testosterone produced displacement curves parallel to the standard curve. The detection limit of the assay (mean ± SD) was 0.3 ± 0.2 nmol/L. The intra-assay coefficients of variation for three control samples were 7.3% (4.4 nmol/L), 5.6% (19.5 nmol/L), and 10.3% (40.6 nmol/L). The corresponding inter-assay coefficients of variation were 10.1, 9.6, and 8.9%.

Testis weight and morphometry. The birds were killed by neck dislocation on the same day as blood was sampled for testosterone analysis. Body weights and testis weights were recorded. Gonado-somatic index (GSI = 100 × testis weight/body weight) and testis weight asymmetry (left testis weight/right testis weight) were calculated. The gonads were fixed in 4% buffered formalin for histologic examination. From the same part of each left testis, a piece was excised with a razor blade. Each sample was embedded in paraffin wax (testes from EE_2-treated birds) or hydroxyethyl methacrylate (testes from DES-treated birds). Sections (4 µm) were prepared from the paraffin-embedded testes and stained with hematoxylin and eosin. The methacrylate-embedded testes were cut into 2-µm sections and stained with toluidine blue. Four samples from the control group in the dose–response experiment were accidentally lost.

The histology of the left testis was evaluated by measuring the diameter of randomly selected tubules in each testis. The measurements were made using a computer-assisted Nikon Microphot FXA light microscope (Nikon, Tokyo, Japan). Only tubules with visible lumens were measured. Tubules appearing oblique were measured over the short axis of the tubular profile. The diameters of 15–20 tubules in each testis sample were measured, and the average tubule diameter was calculated. All measurements were done "blind."

Dose–response experiment. Various doses of EE_2 (2 or 6 ng/g egg) or DES (6, 19, or 57 ng/g egg) were injected into the yolk of the eggs. The quail were hatched and raised in heterosexual groups until 7 weeks of age, when they were separated into individual cages. Sexual behavior tests were performed the eighth week after hatching. The birds were killed 1–2 days after the behavioral tests, and body weight, plasma testosterone concentration, GSI, testis weight asymmetry, and tubule diameter were determined.

Implant experiment. A dose of 57 ng DES/g egg was injected into the yolk of the eggs. After hatching, the quail chicks were raised in heterosexual groups until 3 weeks of age, when they were separated into individual cages. Four weeks after hatching, a 25-mm long Silastic tube (2 mm i.d., 3 mm o.d.) filled with crystalline testosterone and sealed with silicone glue was implanted subcutaneously in the neck region of each bird. The implants were cleaned with 70% ethanol and then placed in 0.9% saline for 12 hr before implantation. Behavioral tests were performed 1 week after implantation.

Blood samples for determination of plasma testosterone concentrations were collected after the behavioral test. At autopsy, presence of the testosterone implant was verified and body weight and cloacal gland area (defined as longest length × greatest width) were measured.

Statistics. The values for the test end points in the dose–response experiment were statistically analyzed by the Kruskal-Wallis test. When this test proved significant ($p < 0.05$), treated groups were compared with the corresponding control using the Mann-Whitney test. In the implant experiment, we used the Mann-Whitney test to compare the treated group with the control group. Correlation between sexual behavior and plasma testosterone concentration was tested with the Spearman rank correlation test. Differences were considered significant when $p < 0.05$ for two-tailed tests.

Results

Dose–response experiment. Sexual behavior was significantly depressed ($p = 0.0048$) following treatment with 6 ng EE_2/g egg (0.07 µg/egg) (Figure 1A). A dose-dependent decrease was noted following treatment with DES, and the lowest dose causing a significant ($p = 0.045$) effect was 19 ng DES/g egg (0.22 µg/egg) (Figure 2A).

Neither GSI nor tubule diameter was significantly affected by any of the treatments (Table 1). Testis weight asymmetry was significantly increased ($p = 0.0073$) by treatment with 6 ng EE_2/g egg (Figure 1B) but not by any of the DES doses (Figure 2B). Plasma testosterone concentrations in the treated birds did not differ significantly from the control values. However, plasma testosterone levels were numerically lower than the control values at the two highest doses of DES, whereas a slightly lower concentration than the control value was noted at the highest EE_2 dose (Table 1). No correlation between plasma testosterone level and frequency of sexual behavior was found in the EE_2- and DES-treated birds (Figure 1C, Figure 2C). Body weight was not affected in any of the treated groups (Table 1).

Implant experiment. Sexual behavior was significantly reduced ($p = 0.005$) in the testosterone-implanted birds treated *in ovo* with 57 ng DES/g egg. The mean behavioral scores (± SD) were 3.86 ± 1.46 and 1.00 ± 1.80 for controls ($n = 8$) and DES-treated birds ($n = 9$), respectively. The depression of sexual behavior was similar to that in the birds treated with 57 ng DES/g egg in the dose–response experiment. Plasma testosterone levels (mean ± SD) were 2.3 ± 1.6 nmol/L for the controls and 2.9 ± 1.9 nmol/L for the DES-treated birds. Sexual behavior and testosterone concentration in plasma were not correlated. The cloacal gland area was significantly reduced by the *in ovo* treatment with 57 ng DES/g egg (248 ± 52 mm^2 for controls and 191 ± 40 mm^2 for DES-treated birds; $p = 0.036$). Body weight was not affected by the treatment (data not shown).

Discussion

Depressed male sexual behavior was the most sensitive end point studied in the first experiment. EE_2 significantly depressed male sexual behavior at 6 ng/g egg, but with and for DES, a significant decrease was observed at 19 ng/g egg. Moreover, we observed a dose–response relationship for this effect. EE_2 increased testis weight asymmetry at 6 ng/g egg, whereas DES treatment did not. Thus, this effect was not observed consistently, even at the highest dosage level used. The GSI and tubule diameter were not significantly affected by EE_2 or DES, and plasma testosterone concentrations did not differ significantly between the control group and the EE_2- and DES-treated groups. Consequently these end points are not as sensitive as depressed sexual behavior and are therefore not recommended as sole indicators of estrogen exposure.

A daily test period of 2 min was used to determine the sexual activity of the male birds. The length of the test period was chosen on the basis of a preliminary study in which 12 sexually active males were presented to receptive females for 2 min during

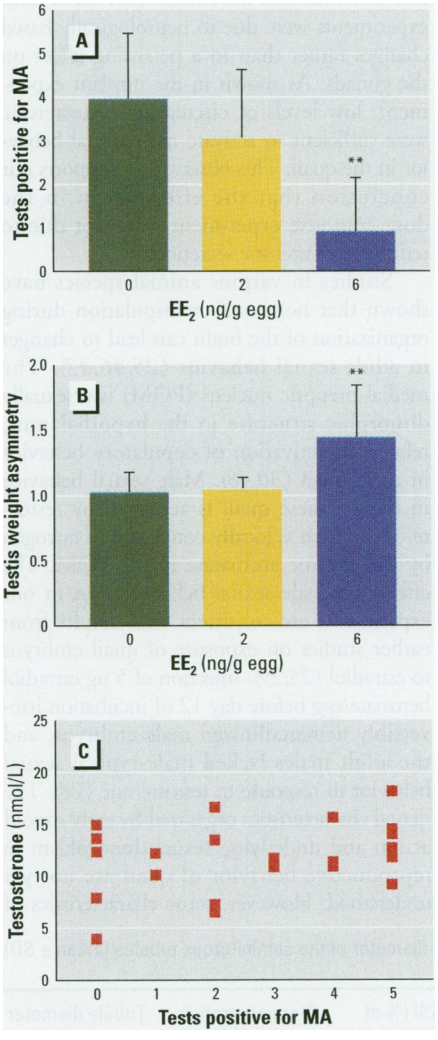

Figure 1. (A) Sexual behavior (mean ± SD), (B) testis weight asymmetry (mean ± SD), and (C) sexual behavior versus plasma testosterone concentration in adult male Japanese quail treated *in ovo* with ethinylestradiol (EE_2). SD, standard deviation. Doses were 0 ($n = 14$), 2 ($n = 5$), and 6 ng/g egg ($n = 7$). Behavioral activity was scored as the number of tests, given 1 per day for 5 consecutive days, during which at least one mount attempt (MA) occured.
**$p < 0.005$.

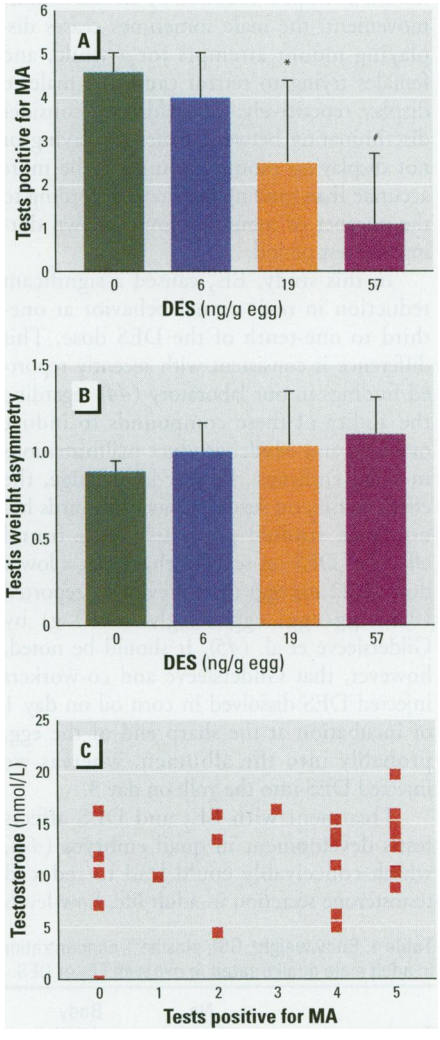

Figure 2. (A) Sexual behavior (mean ± SD), (B) testis weight asymmetry (mean ± SD), and (C) sexual behavior versus plasma testosterone concentration in adult male Japanese quail treated *in ovo* with diethylstilbestrol (DES). SD, standard deviation. Doses were 0 ($n = 8$), 6 ($n = 7$), 19 ($n = 5$), and 57 ng/g egg ($n = 7$). Behavioral activity was scored as the number of tests, given 1 per day for 5 consecutive days, during which at least one mount attempt (MA) occured.
*$p<0.05$. #$p<0.001$.

5 consecutive days, giving a total of 60 opportunities for sexual interaction. At 51 of these, male sexual behavior that consisted of at least one mount attempt was noted. The median latency to the first sexual interaction was 5 sec, with a range of 2–120 sec. A 2-min test period was considered to accurately reflect the sexual activity of male quail. The criteria for deeming males as sexually active were chosen to minimize the risk of bias due to female receptivity. The female may respond to a male displaying neck grab by running away, standing still, or displaying pecking (*35*). As suggested from the preliminary study, a sexually active male is more likely to show cloacal contact movement with a highly receptive female. When a mount attempt is followed by cloacal contact movement, the male sometimes ceases displaying mount attempts for a while, and females trying to retreat cause the male to display repetitively. Therefore we consider discriminating between males displaying or not displaying mount attempts to be more accurate than grading the males according to the number of mount attempts shown during one test period.

In this study, EE_2 caused a significant reduction in male sexual behavior at one-third to one-tenth of the DES dose. This difference is consistent with recently reported findings in our laboratory (*44*) regarding the ability of these compounds to induce ovotestis and Müllerian duct malformations in quail embryos. To our knowledge, the effect of EE_2 on sexual behavior in birds has not been studied previously. We found effects of DES on sexual behavior at a lower dose (0.22 µg/egg) than previously reported (0.48 µg/egg; egg weight 8–10 g) by Gildersleeve et al. (*45*). It should be noted, however, that Gildersleeve and co-workers injected DES dissolved in corn oil on day 1 of incubation at the sharp end of the egg, probably into the albumen, whereas we injected DES into the yolk on day 3.

Treatment with EE_2 and DES affects testis development in quail embryos (*44*), which conceivably could lead to reduced testosterone secretion in adult life. Low levels of circulating testosterone may offer an explanation for the absence of male-typical sexual behavior in birds treated *in ovo* with estrogen-like substances. It should be noted, however, that in our experiments, sexual behavior and plasma testosterone concentration were not correlated, which suggests that the depressed sexual behavior was due to an altered sensitivity of the brain to the activating effects of testosterone rather than to reduced testosterone production by the testes. This suggestion is further strengthened by the results from the implant experiment in which all quail were given a similar endocrine milieu with regard to testosterone. The behavioral effects recorded were similar to those observed in the dose–response experiment. We therefore conclude that the effects observed in both experiments were due to neurologically based changes rather than to a persisting effect on the gonads. As shown in the implant experiment, low levels of circulating testosterone were sufficient to activate male sexual behavior in the quail. This observation supports our conclusion that the effects seen in the dose–response experiment were not due to reduced testosterone secretion.

Studies in various animal species have shown that hormonal manipulation during organization of the brain can lead to changes in adult sexual behavior (*25,46,47*). The medial preoptic nucleus (POM) is a sexually dimorphic structure in the hypothalamus, related to activation of copulatory behavior in male quail (*30,48*). Male sexual behavior in the Japanese quail is activated by testosterone, which is locally converted to estrogen by the enzyme aromatase in the POM. The effects on male sexual behavior seen in our experiments are consistent with results from earlier studies on exposure of quail embryos to estradiol (*25,29*). Injection of 5 µg estradiol benzoate/egg before day 12 of incubation irreversibly demasculinized male embryos, and the adult males lacked male-typical sexual behavior in response to testosterone (*28*). The neural characteristics organized by early steroid action and underlying sexual dimorphism in reproductive behavior of quail are not yet understood. However, some characteristics of the POM, organized by estrogen during the critical embryonic period, have been described. In male quail, vis-à-vis females, the POM is innervated by a dense network of vasotocin-immunoreactive fibers (*49*). Male quail embryos treated with 25 µg estradiol benzoate on day 9 lost the vasotocin immunoreactivity of the POM, simultaneously losing the capacity to display copulatory behavior as adults (*50*). Balthazart (*51*) showed that treatment of male quail embryos with estradiol benzoate on day 9 of incubation depressed preoptic aromatase activity in adulthood. Treatment of male embryos with a dose of estradiol benzoate high enough to reduce adult copulatory behavior also affected neuronal size in the dorsolateral part of the POM in adults (*52*). These characteristics of the POM have been shown to be organized by embryonic treatment with estrogens, and this reinforces the conclusion that the depressed sexual behavior observed in our experiments was most likely due to an organizational effect on the developing brain. It cannot be excluded that estrogen treatment primarily affects the embryonic testis resulting in an increased synthesis of endogenous estrogens, altering the hormonal milieu, and secondarily affecting brain development. However, Scheib et al. (*53*) found that the induced ovotestis does not produce more estrogens than a normal testis.

Exposure to 2 ng EE_2 or 2 ng DES/g egg during sexual differentiation of the gonads induces formation of ovotestes in male quail (*44*). In the present study we did not find histologic changes in the testes of the adult birds. It has been shown that an ovotestis induced in a quail embryo may not persist until adulthood (*54*); this is consistent with our results. However, the persistence of ovotestes may be dose dependent, and in ongoing studies we are examining the histology of the testes of estrogen-treated birds at different time points from hatching until sexual maturity.

There is normally a close correlation between testis weight and the number of germ cells in the testis (*55*). Generally, there is also a positive relationship between the diameter of the seminiferous tubules and the spermatogenic activity of the testis (*56*). In the adult birds in our first experiment, no significant changes in GSI or tubule diameter were seen following exposure to DES or EE_2. This finding indicates that testicular function was not affected in this experiment. However, ultrastructural examination of the testes and studies on sperm viability would be required to finally exclude an effect on testicular function. Tubule diameter in the EE_2-treated groups and their controls differed from the diameter in the DES-treated groups and their controls. This discrepancy was due to the embedding of the testes in metacrylate and paraffin wax, respectively.

Table 1. Body weight, GSI, plasma T concentration, and diameter of the seminiferous tubules (mean ± SD) in adult male quail treated *in ovo* with EE_2 or DES.

Treatment	No. males	Body weight (g)	GSI (% of body weight)	T concentration (nmol/L)	Tubule diameter (mm)
Control for EE_2	14	158 ± 12	2.9 ± 0.5	12.7 ± 2.3	0.23 ± 0.02
EE_2 (2 ng/g egg)	5	155 ± 12	3.1 ± 1.0	13.7 ± 3.5	0.22 ± 0.01
EE_2 (6 ng/g egg)	7	155 ± 13	2.9 ± 0.4	9.8 ± 3.6	0.24 ± 0.03
Control for DES	8	176 ± 11[a]	2.9 ± 0.6[a]	13.7 ± 3.3	0.31 ± 0.02[b]
DES (6 ng/g egg)	7	171 ± 20	2.8 ± 0.4	14.1 ± 2.6	0.28 ± 0.02
DES (19 ng/g egg)	5	172 ± 18	2.6 ± 0.7	11.9 ± 6.2	0.29 ± 0.04
DES (57 ng/g egg)	7	169 ± 14	3.0 ± 0.7	9.5 ± 5.4	0.31 ± 0.03

Abbreviations: DES, diethylstilbestrol; EE_2, ethinylestradiol; GSI, gonado-somatic index; SD, standard deviation; T, testosterone.
[a] $n = 5$. [b] $n = 4$.

The cloacal gland is a foam-producing, androgen-dependent structure that becomes demasculinized by embryonic exposure to estrogen, such as estradiol benzoate (*25*). The reduction in cloacal gland area noted in DES-treated birds in our second experiment is consistent with previous findings after *in ovo* treatment with estrogen (*25,28*). We therefore suggest that measurement of the cloacal gland area should be included as an end point when testing contaminants for estrogenic effects in birds.

EE_2 and DES treatments were performed on separate occasions, and the frequency of sexual behavior in the controls differed on these two occasions. The reason for this is probably naturally occurring variations within the controls. Schein et al. (*57*) reported marked variability in sexual performance levels both among and within male Japanese quail. The importance of large control groups in this type of behavioral experiment should be emphasized.

In conclusion, studying altered sexual behavior in the Japanese quail requires limited resources and can be done in a relatively short time because of the rapid maturation of the species. We plan to perform behavioral studies in our laboratory with environmental contaminants that are suspected estrogens; the potencies of EE_2 and DES presented in this paper will serve as valuable comparisons with potencies obtained in future studies. The *in vivo* test described here took a total of 11 weeks from incubation and included effects on an environmentally important variable (sexual behavior). Chemicals other than estrogen receptor agonists that affect neurochemical systems involved in sexual behavior can also be studied in the test system described. For example, dopamine agonists/antagonists (*58,59*) and aromatase inhibitors (*60,61*) have been shown to affect sexual behavior in the quail. Thus, sexual behavior in the quail is an end point that can be used in testing a variety of environmental contaminants in the search for neuroendocrine-disrupting contaminants.

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