ABSTRACT

Title of dissertation: EFFECTS OF EMBRYONIC EXPOSURE TO
                           ANDROGEN-ACTIVE ENDOCRINE DISRUPTING
                           CHEMICALS IN JAPANESE
                           QUAIL

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Endocrine disrupting chemicals (EDCs) are compounds that alter the production,

secretion, action, and elimination of endogenous hormones. EDCs have been shown to

be responsible for disrupting development, reproduction, immune function, behavior, and

all other life functions mediated by hormones. In the environment, organisms are

exposed to many different types of EDCs at any one time, each with different

mechanisms of action, many of which are not fully understood at present. Most research

done with EDCs has focused on the effects of these chemicals on the estrogen and

thyroid systems, however, many of these same chemicals also exert strong effects on the

androgen system. Also, many studies assessing the effects of EDCs on wildlife have

focused on reproductive measures of exposure, often overlooking potential effects on the

immune system. We have demonstrated that embryonic exposure to androgen-active

EDCs, anti-androgenic DDE and androgenic trenbolone acetate, impairs development of

the bursa of Fabricius in Japanese quail, providing a possible mechanism for EDC-induced immunosuppression. The bursa is a primary immune organ responsible for development of the humoral part of the immune system. We have also demonstrated that the bursa can be resilient to embryonic exposure to EDCs, if post-hatch exposure to these chemicals is prevented. Measures of reproduction, behavior, growth, and developmental stability were also taken in this study. Male and female rates to sexual maturity were altered by the one-time *in ovo* exposure to DDE and trenbolone. Male reproductive behavior, as measured by attempts to mount and successful cloacal contacts achieved, was suppressed by both chemicals. Vocalization was abolished in one and two week old chicks from the highest trenbolone acetate treatment levels. Although environmentally relevant, the levels of DDE used in this study were below those reported to affects avian reproduction. Environmental levels of trenbolone acetate are unknown, however, previous studies have concluded trenbolone acetate to be safe to wildlife and non-teratogenic. The myriad of endpoints used in this study has been compiled to provide toxicologists with a list of sensitive and persistent measures that can be used as reliable biomarkers of exposure to androgen-active EDCs in birds.

EFFECTS OF ANDROGEN-ACTIVE ENDOCRINE DISRUPTING CHEMICALS IN
JAPANESE QUAIL

By

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I dedicate this dissertation to my family, which has blessfully grown this year following my marriage to my beautiful wife Jennifer. When I could not see the light at the end of the tunnel, you were there to remind me where it was and how close I was to this journey's end. Your love and support holds more value than any degree ever could.

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Chapter 1: Introduction

BACKGROUND

Endocrine disrupting chemicals (EDCs) are compounds that alter the production, secretion, action, and elimination of endogenous hormones when encountered in the environment. They are responsible for disrupting development, reproduction, immune function, behavior, and all other life functions mediated by hormones. Most research done with EDCs to date has focused on the effects of these chemicals on the estrogen and thyroid systems. Also, since early studies focused on the effects of estrogenic compounds, traditional research with EDCs had used mainly reproductive measurements. Therefore, until recently, many other organ systems have been largely ignored in the field of toxicology. An overwhelming amount of evidence has since shown a large amount of "crosstalk" to exist between the endocrine and other organ systems, revealing both subtle and vital modulatory roles for many hormone systems in the development and maintenance of the body's organ systems. This dynamic connection between endocrine systems and the rest of the body creates the potential for EDC exposure to disrupt the development and functioning of a myriad of functions.

Toxicology is a relatively new discipline, especially with consideration of compounds that target endocrine systems. Although many observational studies have correlated EDC exposure with a wide variety of biological effects, the mechanisms behind these effects remain elusive. Laboratory studies have been successful in establishing causality with various classes of EDCs. However, the difficulty in being able to accurately document and predict effects largely come from organisms being exposed to many different types of EDCs at any one time in the environment. Each class

of EDCs has its own mechanism of action, many of which are not yet fully understood. Individual EDCs also have more than one potential mechanism of action, and exposure to multiple EDCs may have additive or multiplicative effects, making the link between cause and effect even more difficult to ascertain.

This dissertation was initiated as an investigation of the effects of androgen active EDCs on the development and function of the avian immune system. As reproduction, growth, and behavioral data were also monitored to determine if there were associations between effects, we have gathered a great deal of data which has revealed some striking and unexpected effects. Just as androgen active EDCs are often overlooked in favor of estrogenic and thyroid active EDCs, birds have been overlooked in favor of studies using mammals. Therefore, since there is a scarcity of available data on the effects of androgen active EDCs on the development and activation of avian systems, the opportunistic data taken together with the intended immune response data provides a timely report on the interactions between these chemicals and biological systems. This study also further illustrates how finely interconnected and stongly influenced many systems are with the neuroendocrine systems. Currently, a call has gone out to the scientific community to help establish reliable endpoints, or biomarkers, of EDC exposure in birds. The results of this study will contribute to the identification of sensitive and reliable biomarkers of androgen active EDC exposure in Japanese quail. Two types of environmentally relevant androgen active EDCs were used in this study: an androgenic compound, trenbolone acetate, and an anti-androgenic compound, p,p'-DDE, to discern effects of both types of androgen disruption.

ENDOCRINE INTERACTIONS WITH SYSTEMS STUDIED

A. Immune system

It is well known that many hormones influence the development of lymphoid organs and cells, and that many lymphoid cells secrete a wide variety of hormones and cytokines that interact to some degree with most organ systems. Neuroendocrine and immune system "crosstalk" is discussed in detail by Marsh and Scanes (1994). So much of this crosstalk occurs that the thymus and bursa of Fabricius are sometimes referred to as endocrine organs as well as immune organs. The avian endocrine and humoral immune systems appear to communicate via gonadal steroid receptors on bursal epithelial cells and glucocorticoid receptors on bursal B cells (Sullivan and Wira, 1979). Therefore, it is not surprising that androgens and glucocorticoids exert strong effects on the development and functioning of the bursa. Further evidence for the bursa's role as an endocrine organ is found in the effects of the bursa's main hormonal/growth factor product, bursin, on the immune and adrenocorticotropin systems.

Sex differences in immune response have been observed in many animals (Zuk, 1990). In general, females tend to be more resistant to infection than males. Females also have a greater ability to reject skin grafts and are more efficient in antibody production in response to antigen challenges (Novotny et al., 1983). Three explanations have been hypothesized by evolutionary biologists for these sex differences in immune function (Moller et al., 1998). First, activities related to sexual selection impose a resource drain on males that reduce the resources available for immune function; i.e. energy and nutrients that are spent on courtship and mating cannot be used to prevent or

fight infection. Second, males may have higher levels of potential exposure to endo- and ectoparasites than females. This higher level of exposure may be associated with increased interactions between males in territory defense and matings with more than one female in polygamous species. Lastly, Folstad and Karter (1992) proposed that higher levels of androgens in males responsible for the development of secondary sexual characteristics and sexual displays might negatively impact immunocompetence. Conversely, just as higher levels of androgens in males have been found to be immunosuppressive, estrogens appear to enhance immune responses in females (Novotny et al., 1983).

Many studies have shown that androgens often have a strong immunosuppressive effect in many species of birds. Serum lysozome activity, an index of macrophage activity, is reduced in birds treated with testosterone (Al-Afaleq and Homeida, 1998). Testosterone treatments also resulted in a reduction in the total number of leukocytes and lymphocytes in developing chicks. The first clue of an interaction between androgens and humoral immunity was the negative relationship found between testicular maturation and bursal regression; the bursa begins to regress as the testes mature and steroidogenesis increases. Moreover, the bursa is exquisitely sensitive to testosterone (Glick, 1983), compared to the other primary lymphoid organs, such as the spleen and thymus, which appear to be unresponsive to exogenous androgens (Al-Afaleq and Homeida, 1998).

Administration of many different types of androgens, including androsterone, androstene-3, 17-dione, methylandrostene diol, 5α-dihydrotestosterone (5α-DHT), testosterone propionate (TP), and 19-nortestosterone, disrupts embryonic development of the bursa (Glick, 1980). DHT treatments resulted in reduced numbers of proliferating

bursal cells in broiler chicks (Novotny et al., 1983), and were found to stimulate testes growth (Leitner et al., 1996). Bursas from chicken embryos treated with TP at E 3 were considerably smaller than controls on E 10 (Olah et al., 1986). Most of the mesenchymal cells did not differentiate into light and dark cells. A small number of dark cells that did develop were able to enter the epithelium, but lacked the characteristic cytoplasmic granules. These dark cells have been termed "bud inducers" because they are necessary for epithelial bud formation. The few granule-lacking bud inducing dark cells that formed under TP treatment and entered the epithelial tissue were not able to initiate bud formation. Although the epithelium did not appear to be altered by the TP treatment, it did not accept any hemopoietic stem cells for B cell maturation (Olah et al., 1986). Mase and Oishi (1991) found that Japanese quail treated with testosterone developed wrinkled epithelium in the bursas. Also, the plicas, or buds, did not develop and the follicles were empty, rather than being filled with lymphocytes.

Some discrepancies have been found relative to the effects of testosterone on avian immunocompetence. Ros and coworkers (1997) have shown that exogenous testosterone enhanced antibody titers in response to sheep red blood cells (SRBC) in black-headed gull chicks, but caused no effect on antibody titers in 9 mo old juveniles. One explanation for these effects is that testosterone may not have as strong of an effect on the amount of antibodies produced as it does on the types of antibodies produced. Testosterone has a negative effect on IgG production, and testosterone treated chicks have been found to have higher levels of IgM than controls (Deyhim et al., 1992). The inhibition of affinity maturation by testosterone may prevent normal isotype switch of IgM production to IgG production by lymphocytes. Although the overall amount of

antibodies produced in response to foreign antigens may not significantly decrease under testosterone treatments, the antibodies produced may not be specific to the particular antigen.

The bursa of Fabricius is a lymphoid organ unique to birds that is responsible for B-lymphocyte maturation, the leukocytes responsible for antigen production (Glick et al., 1956). Hieronymus Fabricius first described the bursa as "a cavity...which is situated near the podex and connected with the uterus" and "a double sac which in its lower portion projects toward the pubic bone and appears visible to the observer as soon as the uterus...presents itself into view" (Adelmann, 1967; Glick, 1980). More succinctly, it is a dorsal diverticulum or cul-de-sac of the proctodeal region of the cloaca (Glick, 1983). Because of its location in the avian body, being close to the testes, ovaries, colon, and intestines, some early suggested functions included: semen reservoir, egg reservoir, seminal vesicle, Cowper's gland, anal pouch, urinary vessel, bladder, and third caecum. Thanks to a serendipitous experiment performed by one of Glick's graduate students, we now know that its true purpose deals with the immune system rather than that of digestion, reproduction, or excretion (Glick, 1991). The bursa directs the differentiation of lymphocyte precursor cells into immunologically competent B cells (Assenmacher, 1973). It acts as a primary lymphoid organ during early stages of development, but later regresses into either a peripheral lymphoid organ or a non-functional remnant organ, depending on the species (Pasanen et al., 1998). In precocial birds, the bursa increases to maximum size between the fifth and twelfth week of age, and then regresses before sexual maturity (Glick, 1977). In the Japanese quail, the bursa remains throughout adulthood, possibly serving as a secondary lymphoid organ.

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As suggested by its function in B cell development, the bursa's main role in immune function is humoral. The avian humoral branch of the immune system protects birds from foreign pathogens through the production of antibodies, which is dependent on the normal development of the bursa (Glick, 1973). Early studies demonstrated that antibody production was suppressed in bursectomized birds, suggesting that immunoglobulin synthesis was solely dependent on the bursa (Long and Pierce, 1963; Kincade et al., 1970). However, further research revealed that although immunoglobulin production is reduced in bursectomized birds, there is not complete inhibition of antibody production (Glick, 1991). Embryonically bursectomized birds are able to produce serum and surface immunoglobulin (Ig) M, IgG, and IgA class immunoglobulins, although they cannot make specific antibodies even after heavy immunization (Jalkanen et al., 1984; Guellati et al., 1991). The degree of antibody suppression is dependent on the timing of bursectomy. When normal maturation of B cells is allowed to occur, immunoglobulin synthesis also takes place in the spleen and intestinal mucosa (Thorbecke et al., 1968; Bienenstock et al., 1973). However, during embryonic development, immunoglobulin synthesis is solely dependent on the bursa (Seto, 1981).

Another vital function for the formation of a healthy immune system that is dependent on proper development of the bursa is the creation of antibody diversification. One of the main ways to achieve this is through diversification of immunoglobulin molecules by gene conversion of variable gene segments of the immunoglobulin heavy and light chain genes. This occurs only during the early stages of development and appears to be dependent on the bursa alone (Masteller and Thompson, 1994). The bursa

is also the site of antibody isotype switch, the conversion of one antibody class to another from the rearrangement of constant regions in the B cell heavy chains.

The bursal follicles are the sites of B cell maturation. They develop from endothelial buds, and are made up of a cortex and medulla. The cortex contains lymphoblasts, lymphocytes, macrophages, and plasma cells. In addition to the above described contents, the medulla also contains reticular cells (Olah et al., 1975; Glick, 1983). The formation of the endothelial buds and the maturation of blood borne lymphoid stem cells into immunocompetent B cells are dependent on the mesenchyme. Hemopoeitic cells first migrate to the mesenchyme from the blood stream, but before this happens, mesenchymal cells must develop into either a cell type with a large pale nucleus with one or two small nucleoli (light cells), or a cell type with a polygonal nucleus with a large nucleoli that stains heavily (dark cells). This differentiation of the mesenchymal cells occurs at embryonic day (E) 8 in the chicken (Olah et al., 1986). At E 12, some dark cells begin to move toward an epithelial niche where they begin to produce small dark granules in their cytoplasm. They invade the epithelium that immediately responds by forming a bud. More dark cells surround the bursal bud as it grows and matures. The fully formed bursal follicle is then able to receive blood borne lymphoid stem cells for lymphocyte maturation to take place (Olah et al., 1986).

There is literature that links exposure to EDCs with immunosuppression in birds (review in Crisp et al., 1998). Planar halogenated aromatic hydrocarbons, such as polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin have been shown to have immunotoxic effects (Grasman and Fox, 2001), 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 (Fox and Grasman, 1999). PCBs and dioxins have also been linked to thymic atrophy and suppression of mitogen-induced proliferation, mixed lymphocyte responses, cell-mediated cytotoxicity, delayed-type hypersensitivity reactions, and humoral responses (reviewed in Grasman and Fox, 2001). However, while these studies have established the association of selected EDCs with effects in laboratory and field birds, much more data are needed to establish the extent of impact from EDCs and the classes of these compounds that are most deleterious.

However, even these laboratory studies have their limits when attempting to establish links between individual chemicals and their immunotoxic effects. Many individual EDCs, including polychlorinated biphenyls (PCBs) and pesticides such as methoxychlor (MXC), have more than one mechanism of action. Until recently, people have focused on the estrogenic and thyroid effects of these compounds, even when previous studies have shown these same chemicals to have strong effects on the androgen system. As a result, the androgenic properties, which have almost always been thought of as being immunosuppressive, have often been overlooked.

B. Reproductive System

In avian species, gonadal differentiation occurs during early embryonic development. A sexually dimorphic pattern of gonadal steroids that are produced during this time helps to modulate the sexual differentiation of accessory sex structures and neuroendocrine systems that regulate endocrine and behavioral components of reproduction (reviewed in Ottinger et al., 2001). This, as well as differentiation of the

hypothalamic-pituitary-gonadal (HPG) axis, occurs mainly during late embryonic and early posthatch development (Ottinger 1989). This "critical period" later determines not only male or female endocrine patterns, but sexually dimorphic behavioral patterns as well (Ottinger et al., 2001; Ottinger, 1989). The feedback system between gonadotropin-releasing hormone (GnRH) and gonadal steroids is initiated by embryonic day (ED) 10. Changes in the relative ratio of the concentration of testosterone to that of estradiol play more of a role in sexual differentiation of the HPG axis than absolute amounts of either individually (Ottinger 1989). This ratio could be altered by exposure to androgen active EDCs, thereby altering the feedback system between GnRH and the gonadal steroids.

Male reproductive behavior is often used as a measurement of the influence of gonadal steroids on the neuroendocrine development in Japanese quail. Recently, methods to quantify differences in female reproductive behavior have been successfully tested (Domjan et al., 2003), however, this measure has not yet been used in great frequency. Copulatory behavior in males is controlled by the sexually dimorphic medial preoptic area (POA) or nucleus (Balthazart et al., 2000). Many studies have elucidated the early embryonic changes in the anatomy and neurochemistry of the POA that set into place the mechanisms behind the activation of adult reproductive behavior (reviewed in Castagna et al., 1999). Male Japanese quail copulatory behavior has been experimentally demasculinized by embryonic exposure to either estrogen or androgens (Ottinger et al, 2001; Schumacher et al, 1989). Again, this suggests that the development of these mechanisms might also be modulated by a balance of both gonadal steroids. Embryonic exposure to exogenous androgens has already been shown to disrupt development of the POA. Although the size of the POA is unaltered by exogenous androgens, the size of the

neurons and the volume of their nuclei in the dorsolateral area of the POA become permanently altered (Castagna, 1999; Panzica et al., 1999).

Exposure to exogenous gonadal steroids and aromatase inhibitors have been often shown to alter the development of the avian reproductive system. Embryonic exposure to exogenous estrogen has been shown to cause feminization in male Japanese quail, resulting in persistent Mullerian ducts and incomplete differentiation of the gonads as exhibited by ovotestes (Abdelnabi et al., 1997). Aromatase is the enzyme responsible for the conversion of testosterone to estradiol. Embryonic exposure to aromatase inhibitors, such as tamoxifen and fadrozole, has been shown to defeminize the ovary and accessory structures (Ottinger and vom Saal, 2002). In chickens, females developed bilateral testes that were capable of complete spermatogenesis (Elbrecht and Smith, 1992). These females not only had the physical appearance of males, but exhibited the behavior of males as well.

Since most of the sexual differentiation of the reproductive tract occurs by the last third of embryonic development, timing of steroid exposure, and thus EDC exposure, plays an important role in the resulting effects. For example, embryonic exposure to estradiol or testosterone by embryonic day (ED) 12 results in sex differences in adult male or female behavior (Aste et al., 1996). Administration of either of these treatments after ED12, however, is ineffective in altering adult sexual behavior in Japanese quail (reviewed in Ottinger and vom Saal, 2002).

C. Vocalization

Very few studies have investigated the effects of gonadal steroids on vocalization in non-singing avian species. Testosterone, androstenedione, and 5α-dihydrotestosterone have induced calling in adult male Japanese quail (Adkins and Pniewski, 1978; Wada 1982; Wada 1984). Other studies have demonstrated testosterone's ability to stimulate vocalization in ring doves and pigeons (Pietras and Wenzel, 1974; Cheng and Lehrman, 1975). These experiments had only investigated the activational effects of implanted or injected androgens on adult vocalization. The mechanisms behind steroid effects on the activation of vocalization is not yet fully understood, although two main steroid sensitive areas exist: the brain and the syrinx, or avian voice-box.

The intercollicular nucleus of the mesencephalon is the part of the vocal neural system responsible for producing the distress call (Yazaki et al., 1997), the type of vocalization assessed in this dissertation, in Japanese quail chicks. Adult male neurons from the intercollicular nucleus have many more dendrites than those of females, suggesting that the development of these neurons may be influenced by differences in sex steroids between the sexes, although it has not yet been determined if this difference is indeed caused by testosterone (Yazaki et al., 1999). Testosterone has been shown to affect this vocal neural system during development, modulating the amplitude, frequency, and behavior of the adult male crow. Furthermore, following electrical stimulation of the intercollicular nucleus, females were able to produce the male crow four days after subcutaneous implantation of testosterone (Yazaki et al., 1999). It is known that testosterone can alter the structure of the intercollicular nucleus in adults and induce male

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crowing, however, it is unknown if testosterone is able to modulate this area of the brain for production of the distress call in chicks.

Syrinx mass has been shown to be greater in male zebra finches than females (Lohmann and Gahr, 2000), again suggesting a possible role of sex steroids during development. The zebra finch syrinx has been found to contain androgen receptor mRNA by embryonic day 10, presumably in order to prepare the chick for post-hatch food begging behavior (Godsave et al., 2002). One study has shown that Silastic implants of testosterone in adult zebra finches caused significant increases in syrinx mass and the size of the ventralis and dorsalis syrinx muscles, and implants of flutamide, an anti-androgenic chemical, decreased syrinx weight (Wade and Buhlman, 2000). However, results from the few studies that investigate the effects of gonadal steroids on syrinx development are inconsistent. It is clear that the effects of these hormones on syrinx development is complicated, and it is generally agreed that they do not appear to be directly responsible for the stimulation and control of the overall process of sexual differentiation of the syrinx. However, it should be noted that areas of the forebrain that lead to the syrinx can be altered by exposure to exogenous gonadal steroids (Wade et al., 2002).

CHEMICAL TREATMENTS

A. Trenbolone Acetate

Many studies have shown sex hormones released from human and animal wastes to have endocrine-disrupting effects, and have most often focused on the effects of

natural and synthetic estrogens (Tilton et al., 2002; Metcalfe et al., 2001; Purdom et al., 1994). A lot of attention has been directed towards the synthetic estrogens since they are more environmentally stable and more resistant to microbial degradation than the natural steroids (Tabak et al., 1981; Tabak and Bunch, 1970). Fewer studies have looked at possible endocrine-disrupting activities of androgens from human and animal excreta, and as such, are less clear. Trenbolone acetate (17β-acetoxyestra-4,9,11-triene-3-one) is a synthetic androgen that is used in many meat-exporting countries. Upon release into the blood from subcutaneous implants usually in an animal's ear, trenbolone is almost immediately hydrolyzed to the active trenbolone-17β (TbOH-17β; Schiffer et al., 2001). Only one metabolic route has been observed in the heifer, oxidation of TbOH-17β to trendione (TbO), followed by reduction to TbOH-17α. This epimerization between TbOH-17β and TbOH-17α can go back and forth until the molecules are excreted. TbOH-17β is the compound responsible for most of the anabolic activities since the potency of TbOH-17α is only 5% of that of TbOH-17β (Pottier and Cousty, 1981). The affinity of TbOH-17β to the recombinant human androgen receptor is similar to dihydrotestosterone, and that of TbOH-17α is only 5% of the TbOH-17β value (Bauer et al., 2000).

The half-life of TbOH's 17α isomer in liquid manure was found to be 267 days, and that of the 17β isomer is 257 days (Schiffer et al., 2001). Also, TbOH was traceable in soil fertilized with solid dung collected from cattle that received trenbolone implants for up to 58 days. The long half-life of TbOH and its persistence in soils treated with manure from animals given trenbolone implants, create the potential for this synthetic androgen to accumulate in soils and in higher trophic levels of food webs. The potential

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for problems is evident when considering that in the US alone, several tons of trenbolone acetate are applied each year (Schiffer et al., 2001).

B. p,p'-DDE

DDE, or ethylene, 1,1-dichloro-2,2-bis(p-chlorophenyl), is the main metabolite of the pesticide DDT, or 1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane. DDT was used widely as a potent contact poison against arthropods from the 1940s until its ban in most major industrial countries in the 1970s. A few countries still use DDT in small quantities to help control the spread of diseases such as typhus and malaria (Raloff, 2000). Since its ban, levels of DDT have been steadily declining, however DDE still remains relatively high in the world's environmental and organismal systems, as it is chemically more stable and biologically more persistent than its parent compound. DDE is only found in the environment as a result of contamination or breakdown of DDT.

DDT and its metabolites are all lipid soluble compounds, being distributed to all body tissues via the blood and lymph, stored in proportion to organ lipid content (Clement, 1994). In the mammalian body, DDT is broken down to two main metabolites, DDD [1,1-(p-chlorophenyl)-2,2-di-chloroethane] and DDE, before being further broken down to the excretory metabolite, DDA [bis(p-chlorophenyl)acetic acid]. In Japanese quail, the two main metabolites are DDE (31%) and DDA (35%); DDD has been reported to have been found as an avian metabolite of DDT, but is thought to arise postmortem, produced by anaerobic reductive dechlorination by bacteria in the gut or feces (Ahmed and Walker, 1979; Jefferies and Walker, 1966). As stated above, DDE is chemically more stable and biologically more persistent than its parent compound. The ranking of

biological half-lives and affinity for storage in lipid tissue is: DDE>DDT>DDD; some scientists speculate that the *in vivo* persistence of DDE is so high, that most of the DDE that enters the body or is created in the body by metabolism of DDT remains in adipose tissue until death (reviewed in Clement, 1994). After Phase I metabolism in mammals, DDA is excreted through the urine mostly in conjugated form. Some debate exists as to how much of what particular metabolites are excreted in urine and feces; this is further complicated in the bird because urine and feces are combined in the droppings. In both cases, however, the main end metabolite remains DDA.

It took about 50 years after the initial use of DDT as a pesticide to understand some of the mechanisms of action of its main metabolite DDE. This is due in part to much of the early research concentrating on the estrogenic effects of o,p'-DDT, which only made up about 15% of technical DDT (Clement, 1994). o,p'-DDT was shown to cause estrogenic effects in female rats. It wasn't until studies began to show phenotypic effects of this chemical on male rats that were very similar to those observed with exposure to known androgen receptor blocking chemicals that scientists first began to speculate about p,p'-DDE's antiandrogenic nature (Kelce et al., 1995). It is now known that DDE is a potent androgen receptor antagonist and a very potent testosterone hydroxylase modulator. Its androgen receptor blocking ability is almost equal to that of the anti-androgen hydroxyflutamide (Kelce et al., 1995). Moreover, its effects on enzymatic activities are considered secondary mechanisms of action, which are also anti-androgenic. DDE has been shown to induce 6β-, 16β-, and 17β–testosterone hydroxylase in rats. 17β- testosterone hydroxylase acts in the liver to convert testosterone to androstenedione, a less potent androgen (You, 2000). It has also been shown to cause

increases in hepatic aromatase, which could further decrease levels of testosterone through its conversion to 17β–estradiol.

Chapter 2: Effects of DDE on the Immune System

INTRODUCTION

Environmental contaminants are known to be immunotoxic to a number of different taxa, however few studies have assessed the relationship of contaminants and immune function in birds (Bustnes et al., 2004). The avian neuroendocrine system controls many aspects of lymphoid organ differentiation and development and can regulate immediate immune responses (reviewed by Glick, 1984). In turn, immune organs and cells can produce a number of hormones and cytokines that mediate a variety of neuroendocrine responses (Marsh and Scanes, 1994). This intimate interaction between the neuroendocrine and lymphoid systems makes the potential effects of endocrine disrupting chemical (EDC) exposure on these systems of great concern to ecotoxicologists.

P,p'-DDE [ethylene, 1,1-dichloro-2,2-bis(p-chlorophenyl)] is an anti-androgenic metabolite of the pesticide DDT [1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane]. DDE was largely responsible for the decline of many bird populations through severe eggshell thinning (Weseloh et al., 1983). After DDT was banned in 1972, the incidence of eggshell thinning syndrome decreased, and bird populations rebounded (Custer et al., 2000). However, because DDE is highly persistent and lipophilic, great potential continues to exist for bioaccumulation and biomagnification. Current studies show that DDE still has significant effects on avian reproduction and eggshell thinning (Custer et al., 1999; Custer et al., 2000). Current studies also suggest that exposure to DDE may

alter aspects of avian immune function (Grasman and Fox, 1999), however the full extent of these alterations and the mechanisms behind them are largely unknown.

The bursa of Fabricius is a primary lymphoid organ unique to birds that is essential for normal development of the humoral immune system. Restricting its development or removal at hatch drastically reduces immunoglobulin production and depresses plasma cell and germinal center formation. The bursa is extremely sensitive to testosterone (Glick, 1983), compared to the other primary lymphoid organs, such as the thymus and spleen, which appear to be unresponsive to exogenous androgens (Al-Afaleq and Homeida, 1998). Administration of many different types of androgens, including androsterone, androstene-3, 17-dione, methylandrostenediol, 5α-dihydrotestosterone (5α-DHT), testosterone propionate (TP), and 19-nortestosterone, disrupts embryonic development of the bursa (Glick, 1980). Embryonic exposure to exogenous androgens has caused inhibition of the development of bursal buds and follicles, most likely through inhibition of mesenchymal cell differentiation (Olah et al., 1985; Ledouarin et al., 1980). The follicles are areas of the bursa that are responsible for B cell maturation. Inhibition of follicle development may, therefore, result in lasting effects on antibody response quality. Androgens also have been shown to affect the development of the bursal epithelial anlage, resulting in inhibition of acceptance of hemopoietic stem cells that migrate to the bursa in order to undergo B cell maturation.

Since the bursa is exquisitely sensitive to androgens, embryonic exposure to androgen active EDCs should affect its development. The purpose of this study was to assess the effects of an anti-androgenic EDC on the development of the bursa of Fabricius. The first objective of this study was to determine if embryonic exposure to

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DDE would disrupt the development of the bursa's follicles and epithelium. The second objective was to ascertain if this one time embryonic exposure would have lasting effects on immunocompetence throughout adulthood.

METHODS

Japanese quail (*Coturnix japonica*) was chosen as a model species in this study because they are well suited for toxicity tests (Fair et al., 1999). Japanese quail are particularly useful in studies that examine bursal development and function because unlike most avian species, the quail maintains a bursa throughout life (Pardue, 1981). Eggs were collected from a random bred colony at the Department of Animal and Avian Sciences at the University of Maryland, College Park.

One hundred eggs per treatment were randomly assigned to either control (sesame oil vehicle) low DDE (20 ug/egg) or high DDE (40 ug/egg) treatments. All treatments were administered into the yolk at a volume of 20 μl to mimic maternal deposition of the EDC. The low dose reflects environmentally relevant levels that should not affect reproduction, but should have an effect on the development of the bursa; the high dose reflects environmentally relevant levels that have been found to affect reproduction (Nisbet 1989, Henny and Herron, 1989; White et al., 1988; Custer and Mitchell, 1987; Henny et al., 1984; Custer et al., 1983). Holes from the injection site were immediately sealed with paraffin, and the eggs were immediately set to incubate.

Samples were collected from 20 birdsd on day of hatch. Spleens and bursas were collected, weighed, and stored in Bouin's solution. Bursas and spleens were embedded in paraffin, sectioned at 10 μm, and stained with hematoxylin and eosin (according to

Addison, 1929). The slides were then read at 400x and the images were digitized by IPLab for Windows (Scanalytics, Inc., Fairfax, VA). Follicle size was analyzed as an average of 10 follicles per individual bursa. The number of follicles per section was determined by averaging the numbers of follicles from sections taken in 3 different locations along each bursa. Blood was collected to measure serum IgG levels and to make slides for total and differential leukocyte counts. The remaining quail were placed into heated brooders with diet (Purina Gamebird Startina, St. Louis, MO) and water provided *ad libitum*. Sexually mature adults were euthanized and bursas and spleens were collected and processed similarly to those collected from chicks.

Two blood smears were made per individual. The blood was fixed with 100% methanol and stained with Wright stain. The slides were immersed in 100% stain for 30 sec and then placed in a 1:1 dilution of stain in distilled water for 90 sec. The slides were then rinsed with distilled water and allowed to air dry. The total number of leukocytes was determined by counting at 400x magnification and averaging the number of leukocytes found in ten different randomly selected fields. The number of leukocytes per ml of blood was estimated by multiplying this value by 2000. Percents of lymphocytes, monocytes, heterophils, and eosinophils, and the heterophil:lymphocyte (H:L) ratio were also determined by counting 200 leukocytes per individual under 1000x magnification.

The measurement of antibody production in response to foreign red blood cells is a common method for assessing humoral immunity (Fair et al., 1999). Quail were immunized with chukar partridge (*Alectoris chukar*) erythrocytes (CRBCs) because Japanese quail are minimally sensitive to sheep, human, chicken, turkey, and duck erythrocytes and do not produce immunoglobulins to human, bovine, and mouse

albumins (Benton et al., 1977; Pardue, 1981). CRBCs were collected from birds raised at Mason-Dixon Game Outfitters game farm (Pylesville, MD). Adult quail received 0.1 ml of a 1.0% suspension of CRBCs in sterile saline injected into the jugular vein. Serum was collected from the jugular vein one week after the CRBC injection, and serum was stored at -80°. Total (IgM and IgG) and 2-mercaptoethanol-resistant (IgG) antibody activities were measured by microtiter activity methods described by Grasman et al. (1996).

Cell mediated response was measured at 8 weeks of age using a 0.1 ml dose of 1 mg/ml PHA-P (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS). Feathers were plucked from both wing webs. One wing web was injected with the PHA solution while the other wing web received an injection of PBS alone. The thickness of each wing web was measured to the nearest 0.01 mm immediately before and 24 hr after the injections using pressure-sensitive micrometers with a low-tension spring that did not crush the skin (model P.N. 50059, Chicago Brand, Fremont, CA, USA). A stimulation index was calculated as the difference in the change in thickness of the PHA-injected wing web from the change in thickness of the PBS-injected wing web.

Serum IgG was measured in blood collected from day old chicks by an enzyme linked immunosorbent assay (chicken IgG ELISA, Bethyl Laboratories, Montgomery, TX, USA). The ELISA was validated for IgG measurements in Japanese quail by demonstrating parallelism of the standard curve and samples of quail serum. Sensitivity was 1.2 ng/ml and precision, calculated as percent coefficient of variation, was 4.3%.

The Statistical Analysis System (SAS Institute, Inc., Cary, NC) was used for all statistical analyses. Assumptions for parametric statistics were examined prior to

analysis. Data were analyzed separately by sex when sex-by-treatment interactions were significant. All measurements were analyzed by two-way analysis of variance, and Tukey tests were used for post hoc pairwise comparisons.

RESULTS

The bursas of Fabricius from the low and high treated day old chicks were significantly larger than controls (p<0.01; Fig. 1). Spleen weights did not differ significantly. Day old bursas from the control and DDE treatments looked radically different from each other (Fig. 2). The low and high DDE-treated bursas lost the characteristic shape of the folds, or buds, that contain the follicles. Vacuolization of the follicles was also apparent in a number of hatchling DDE-treated bursas, but not in controls. No differences in the appearance of the epithelium were apparent among the treatments. Although the size of the bursal follicles did not change, bursas from DDE-treated birds contained significantly fewer follicles than controls (p<0.05; Fig. 3). In adults, bursas from all treatments no longer exhibited any differences in appearance or in any of the quantifiable measurements. Spleens collected from hatchlings and adults were similar in appearance among all the treatments.

The numbers of total serum leukocytes from day old quail in both the low and high DDE treatments were more than twice as high as those found in birds from the control treatment (p<0.05; Fig. 4). No significant differences were observed with the differential leukocyte count; the numbers of all types of leukocytes increased similarly in the DDE-treated birds. Levels of IgG in hatchling serum did not differ among treatments.
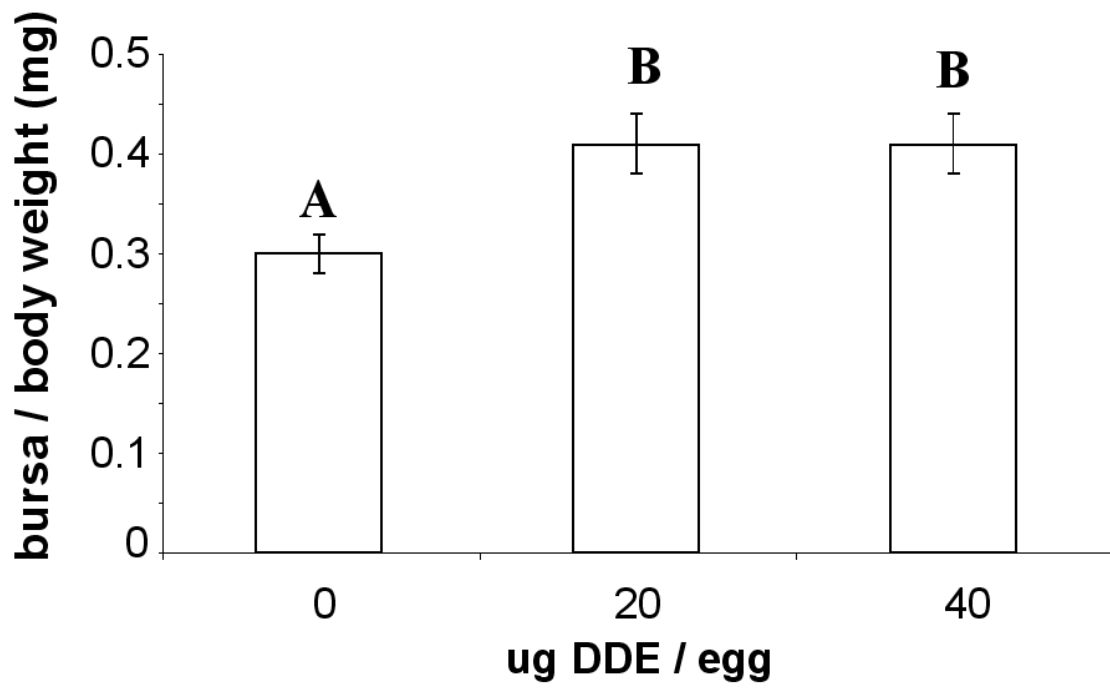
Figure 1. Bursa / body weight indices (mean +/- SEM) in one day old Japanese quail. Significant differences between treatments are indicated by letters (p<0.05).
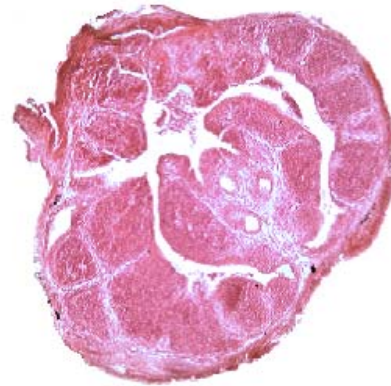
Figure 2. Photographs illustrating the effects of embryonic exposure to DDE on the development of bursas of Fabricius from one day old Japanese quail. Organization of the plicas from bursas collected from DDE treated quail was altered. Vacuolization of the follicles was also apparent in many of the bursas from DDE treated birds.
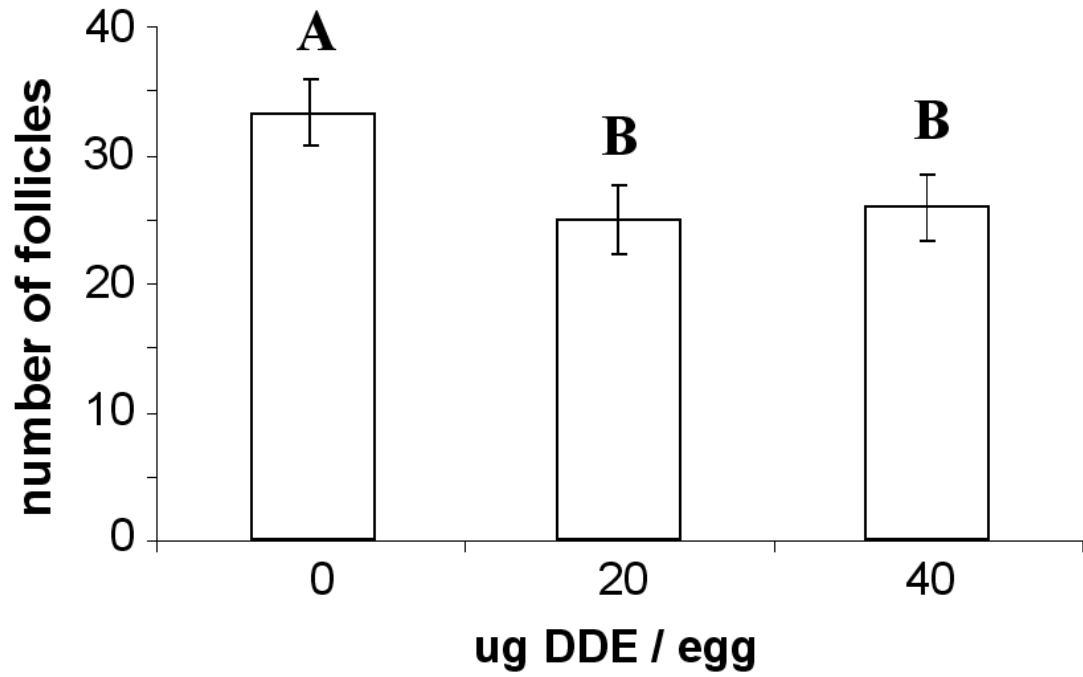
Figure 3. Number of follicles per section (mean +/- SEM) collected from one day old Japanese quail. Significant differences between treatments are indicated by uppercase letters for hatchlings and lowercase letters for adults (p<0.05).
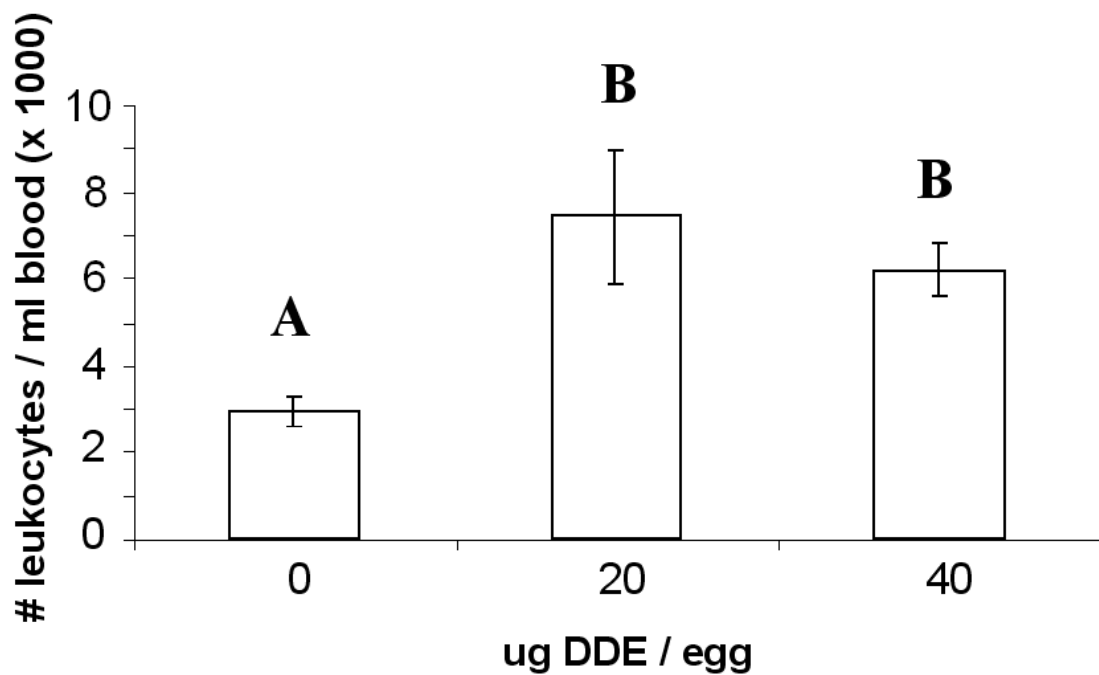
Figure 4. Number of leukocytes per ml blood (mean +/- SEM) collected from one day old Japanese quail. Data was averaged from observations made with two blood smear slides. Significant differences between treatments are indicated by letters (p<0.05).

Although significant differences were observed in regard to the structure and morphology of the bursa, no significant differences in the adult humoral response to a CRBC challenge were observed with either IgM or IgG production. Similarly, although the cell-mediated responses tended to be lower in both adult DDE treatments than controls, these differences were only marginally significant ($p<0.1$).

DISCUSSION

This study has demonstrated that embryonic exposure to DDE, an anti-androgenic EDC, alters the development of the bursa of Fabricius. Previous studies have shown embryonic exposure to androgens to inhibit bursal growth (Meyer et al., 1959; Bruggeman et al., 2003). As hypothesized, embryonic exposure to this anti-androgenic chemical had an opposing effect in developing Japanese quail; natural regression caused by the release of endogenous androgens was reduced in quail exposed to DDE. This reduction of embryonic bursal regression has also been demonstrated in a study using the anti-androgenic chemical flutamide (Burke, 1996). Although the histology from this study showed obvious differences in the appearance of the cross-sections of the DDE-treated and control bursas, finding measurements to quantify these differences was challenging. Since B cells mature in the follicles, it is assumed that disruption in the development of these structures would impact B cell maturation. Vacuoles were found in a number of follicles in bursas that were grown under DDE exposure, however none were found in control bursas. Also, the number of sites of B cell maturation were significantly reduced in DDE-exposed bursas. As one might have expected, we observed a larger leukocyte count in individuals possessing larger primary lymphoid organs.

From observing differences in the appearance of bursal follicles and lymphoid cell numbers, we expected to see differences in the amount of serum IgG in day old quail. All immunoglobulins in day old serum would have come from one of two sources: deposition by the hen as the egg was being formed in the oviduct or production by the embryo. All antibodies that are produced by the embryo at day one only come from B cells that matured in the bursa, whereas after hatch, B cell maturation can occur in other lymphoid organs. Therefore, assuming that maternal deposition of IgG into the eggs was similar among the hens of the colony, any differences that may have been observed would have been due to differences in the chick's B cell population. None were observed, suggesting that although the structure of the bursa was altered by DDE, stem cells were still able to mature within the bursa and produce antibodies.

This measure of hatchling serum IgG was also used to detect possible differences in isotype swich, the conversion of one antibody class to another from the rearrangement of constant regions in the B cell heavy chains. Androgens have been shown to cause increases in IgM and decreases in IgG (Deyhim et al., 1992). These changes may be due to inhibition of isotype switch by androgen disruption of affinity maturation. Since no differences in hatchling serum IgG were observed, it appears that isotype switch was unaffected by embryonic exposure to DDE.

We did not predict differences in the development of the spleen or differences in the cell mediated responses since the avian spleen and thymus are known to be resistant to androgens. We did, however, expect to see differences in the humoral response due to differences observed in the structure of the bursa. After completing the histology of the adult bursas, it was obvious as to why no differences in humoral responses were

observed; without subsequent exposure to DDE post-hatch, the bursas were able to overcome the early developmental disruption and resume normal maturation. Because we were conservative with the age at which the humoral response was conducted, quail were tested when they were immunologically mature. This enabled observation of a healthy response that theoretically would have been large enough for any differences to be observable. In doing so, however, we may have missed testing one of the birds' most immunologically vulnerable stages of life.

These data are encouraging for DDE exposure in wild populations because we did not detect significant differences in the immune responses in adult quail that were exposed to levels of DDE that were strong enough to alter bursal development. This study, however, mimics only maternal deposition of DDE into the yolk. Considering that birds in the wild are continually exposed to this chemical, and a multitude of others, through their diet throughout their entire lives, the full potential of the bursa's resilience to androgen active EDC exposure may not be fully realized in the environment. Further studies are needed to not only test the humoral response in younger birds, but also to test bursa response with continued posthatch exposure to DDE.

It is clear that embryonic exposure to DDE disrupts the normal development of the bursa of Fabricius. Although studies have associated EDC exposure with altered lymphocyte organ development and immune responses, none have yet addressed how this EDC-induced immunosuppression may be occurring. By demonstrating how strong an effect this anti-androgenic contaminant can have on the development of the bursa of Fabricius, this study suggests that disruption of the development of the bursa may be a key factor in the cause of EDC-induced immunosuppression.

Chapter 3: Effects of Trenbolone Acetate on the Immune Sytem

INTRODUCTION

Many studies have shown sex hormones released from human and animal wastes to have endocrine-disrupting effects, and have most often focused on the effects of natural and synthetic estrogens (Tilton et al., 2002; Metcalfe et al., 2001; Purdom et al., 1994). Fewer studies have considered possible endocrine-disrupting activities of androgens from human and animal excreta, and as such, effects are less clear. Trenbolone acetate (17β-acetoxyestra-4,9,11-triene-3-one) is a synthetic androgen that is used as a growth promoter for beef cattle in many countries. Upon exposure, trenbolone is almost immediately hydrolyzed to the active trenbolone-17β, which has an affinity to the recombinant human androgen receptor similar to dihydrotestosterone (Bauer et al., 2000). The half-life of trenbolone and its metabolites can reach over 250 days in liquid manure and be found in soil fertilized with solid dung collected from cattle that received trenbolone implants for about 2 months (Schiffer et al., 2001). The long half-life of trenbolone and its persistence in soils treated with manure from animals given trenbolone implants, create the potential for this synthetic androgen to accumulate in soils and in higher trophic levels of food webs.

The bursa of Fabricius is a primary lymphoid organ unique to birds that is responsible for the maturation of lymphocyte precursor cells into immunologically competent B cells (Glick et al., 1956; Assenmacher, 1973). It acts as a primary lymphoid organ during early stages of development, but later regresses into either a peripheral lymphoid organ or a non-functional remnant organ, depending on the species (Pasanen et

al., 1998). In precocial birds, the bursa increases to maximum size between the fifth and twelfth week of age, and then regresses before sexual maturity (Glick, 1977). In the Japanese quail, the bursa remains throughout adulthood, possibly serving as a secondary lymphoid organ.

This natural regression is initiated embryonically as the testes mature and steroidogenesis increases. The bursa has been described as being exquisitely sensitive to androgens compared to the other primary lymphoid organs, such as the spleen and thymus, which appear to be unresponsive to exogenous androgens (Glick, 1983; Al-Afaleq and Homeida, 1998). Embryonic exposure to exogenous androgens including androsterone, androstene-3, 17-dione, methylandrostene diol, 5α-dihydrotestosterone (5α-DHT), testosterone propionate (TP), and 19-nortestosterone, have been shown to disrupt bursal development as evidenced by bursas with smaller masses, greater number of morphological alterations, and reduced numbers of proliferating cells (Glick, 1980; Novotny et al., 1983; Olah et al., 1986; Mase and Oishi, 1991). B cells are responsible for antibody production, and as a result, alterations in the bursa's development also negatively impacts antibody production. Not only are the numbers of antibodies reduced in birds embryonically exposed to androgens (Bhanushali, 1985; Yoshikawa 1978), but the quality of the fewer antibodies produced may also be compromised, as illustrated by inhibition of normal isotype switch of IgM production to IgG production by lymphocytes (Deyhim et al., 1992; Bhanushali and Ragland, 1985; Hirota et al., 1980).

Many studies have been done to assess potential teratogenic effects of trenbolone, however much of this data was collected in industry laboratories and remains unpublished (Wilson et al., 2002).   The potential for endocrine disrupting problems is

evident when considering that in the US alone, several tons of trenbolone acetate are applied each year (Schiffer et al., 2001). Very few studies have been conducted to assess effects of trenbolone on wildlife, and none have been published to date that investigate its effects in avian species. The purpose of this study, therefore, was to test for endocrine disrupting effects of this androgenic chemical on the development and functioning of androgen-sensitive areas of the immune system. We hypothesized that embryonic exposure to trenbolone would act much like other androgens in causing an inhibition of growth or by causing premature regression of the bursa of Fabricius. We also hypothesized that disruption of natural development of the bursa would cause immunosuppression throughout adulthood, especially in regard to immunoglobulin production and humoral responses.

METHODS

Japanese quail (*Coturnix japonica*) was chosen as a model species in this study because they are well suited for toxicity tests (Fair et al., 1999). Japanese quail are particularly useful in studies that examine bursal development and function because unlike most avian species, the quail maintains a bursa throughout life (Pardue, 1981). Eggs were collected from a randomly bred colony at the Department of Animal and Avian Sciences at the University of Maryland, College Park.

Ninety eggs per treatment were randomly selected to receive either sesame oil (control), 0.05, 0.5, 5 or 50 μg trenbolone acetate (Sigma Chemical Co., St. Louis, MO, USA). Trenbolone was dissolved in sesame oil and injected into yolks at a volume of 20 μl on day four of incubation. Treatments were injected into the yolk to mimic maternal

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deposition. Levels of trenbolone in yolks in the environment are unknown; the levels of

trenbolone used in this study were determined as part of a larger range finding study.

Holes in the egg shell from the injection site were immediately sealed with paraffin, and

the eggs were immediately set to incubate.

Samples were collected from 20 birds on day of hatch. Spleens and bursas were

collected, weighed, and stored in Bouin's solution. Bursas and spleens were embedded in

paraffin, sectioned at 10 μm, and stained with hematoxylin and eosin (according to

Addison, 1929). The slides were then read at 400x and the images were digitized by

IPLab for Windows (Scanalytics, Inc., Fairfax, VA). Follicle size was analyzed as an

average of 10 follicles per individual bursa. The number of follicles per section was

determined by averaging the numbers of follicles from sections taken in 3 different

locations along each bursa. Blood was collected to measure serum IgG levels and to

make slides for total and differential leukocyte counts. The remaining chicks were placed

into heated brooders with diet (Purina Gamebird Startina, St. Louis, MO) and water

provided *ad libitum*. Sexually mature adults were euthanized and bursas and spleens

were collected and processed similarly to those collected from chicks.

Two blood smears were made per individual. The blood was fixed with 100%

methanol and stained with Wright stain. The slides were immersed in 100% stain for 30

sec and then placed in a 1:1 dilution of stain in distilled water for 90 sec. The slides were

then rinsed with distilled water and allowed to air dry. The total number of leukocytes

was determined by counting at 400x magnification and averaging the number of

leukocytes found in ten different randomly selected fields. The number of leukocytes per

ml of blood was estimated by multiplying this value by 2000. Percents of lymphocytes,

monocytes, heterophils, and eosinophils, and the heterophil:lymphocyte (H:L) ratio were also determined by counting 200 leukocytes per individual under 1000x magnification.

The measurement of antibody production in response to foreign red blood cells is a common method for assessing humoral immunity (Fair et al., 1999). Quail were immunized with chukar partridge (*Alectoris chukar*) erythrocytes (CRBCs) because Japanese quail are minimally sensitive to sheep, human, chicken, turkey, and duck erythrocytes and do not produce immunoglobulins to human, bovine, and mouse albumins (Benton et al., 1977; Pardue, 1981). CRBCs were collected from birds raised at Mason-Dixon Game Outfitters game farm (Pylesville, MD). Adult quail received 0.1 ml of a 1.0% suspension of CRBCs in sterile saline injected into the jugular vein. Serum was collected from the jugular vein one week after the CRBC injection, and serum was stored at -80°C. Total (IgM and IgG) and 2-mercaptoethanol-resistant (IgG) antibody activities were measured by microtiter activity methods described by Grasman et al. (1996).

Cell mediated response was measured at 8 weeks of age using a 0.1 ml dose of 1 mg/ml PHA-P (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS). Feathers were plucked from both wing webs. One wing web was injected with the PHA solution while the other wing web received an injection of PBS alone. The thickness of each wing web was measured to the nearest 0.01 mm immediately before and 24 hr after the injections using pressure-sensitive micrometers with a low-tension spring that did not crush the skin (model P.N. 50059, Chicago Brand, Fremont, CA, USA). A stimulation index was calculated as the difference in the change in thickness of the PHA-injected wing web from the change in thickness of the PBS-injected wing web.

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Serum IgG was measured in blood collected from day old chicks by an enzyme linked immunosorbent assay (chicken IgG ELISA, Bethyl Laboratories, Montgomery, TX, USA). The ELISA was validated for IgG measurements in Japanese quail by demonstrating parallelism of the standard curve and samples of quail serum. Sensitivity was 1.2 ng/ml and precision, calculated as percent coefficient of variation, was 4.3%.

The Statistical Analysis System (SAS Institute, Inc., Cary, NC) was used for all statistical analyses. Assumptions for parametric statistics were examined prior to analysis. Data were analyzed separately by sex when sex-by-treatment interactions were significant. All measurements were analyzed by two-way analysis of variance, and Tukey tests were used for post hoc pairwise comparisons.

RESULTS

As hypothesized, the bursa of Fabricius was smaller in birds that were embryonically exposed to trenbolone acetate (Fig. 5A; $p<0.0001$). Bursas that were collected from day old chicks that were exposed to the 5 μg trenbolone were about one-forth smaller than controls ($p<0.05$). Bursas from quail exposed to 50 μg trenbolone were approximately three-forths smaller than controls and half as large as those from birds exposed to 5 μg trenbolone ($p<0.05$). No significant differences in size were observed with bursas collected from adult quail. Although no significant differences

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A.

B.
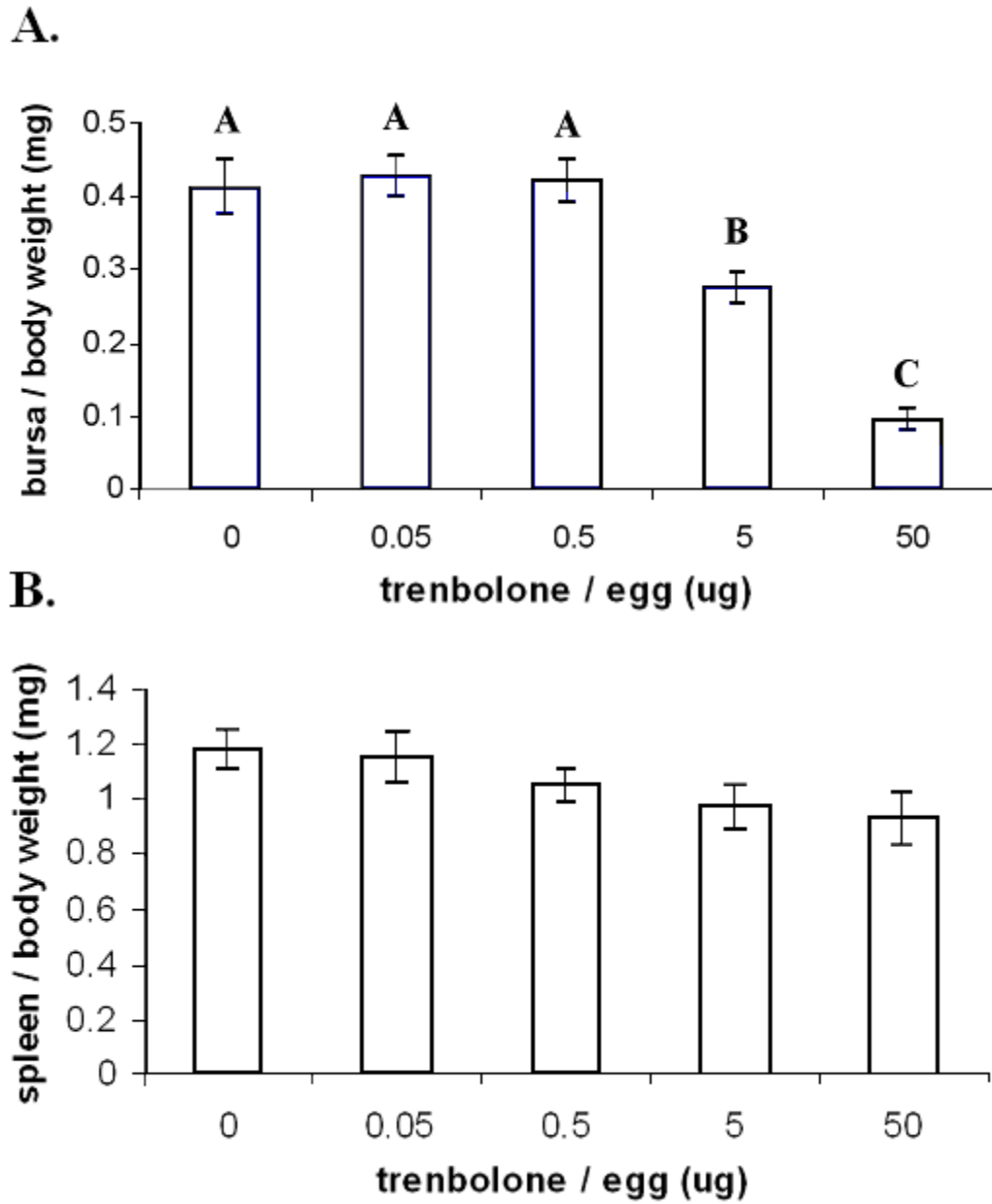
Figure 5. Bursa (A) and spleen (B) / body weight indices (mean +/- SEM) in one day old Japanese quail. Significant differences between treatments are indicated by letters (p<0.05).

were observed in hatchling spleen size across treatments, there was a slight decrease in size as exposure levels of trenbolone increased (Fig. 5B). Spleens from adults were similar across treatments.

The appearance of the bursas from day old chicks grown under exposure to trenbolone was radically different from controls (Fig 6A). The characteristic folds that are apparent in the control bursas were reduced in those treated with trenbolone. Many bursas that were collected from birds belonging to the 50 µg treatment did not appear to have any folds whatsoever. Often, follicles could not be found in the bursas collected from this treatment group. Many of the trenbolone-treated bursas that did have follicles within their folds also exhibited vacuolization within these follicles. The epithelial cell layer that bordered the folds was also much thicker than those observed in control bursas.

Although bursas from the trenbolone-treated quail appeared to develop similarly to controls, some differences were apparent in bursas collected from adult birds (Fig 6B). Compared to controls, the number of follicles found in trenbolone-treated bursas appeared to be reduced and larger in size. Trenbolone-treated bursas also appeared to contain less lymphoid tissue than bursas collected from control birds. The differences in the thickness of the epithelial layers, however, were no longer apparent in the adult bursas.

Quantifiable differences in hatchling and adult bursal morphology existed. There was some variation in the differences in follicle size from hatchling bursas across treatments (Fig 7). Follicles from the 0.05 µg trenbolone treatment were significantly larger than controls ($p<0.05$). Hatchling follicles from the highest treatment level were

A.

B.

control   trenbolone-treated
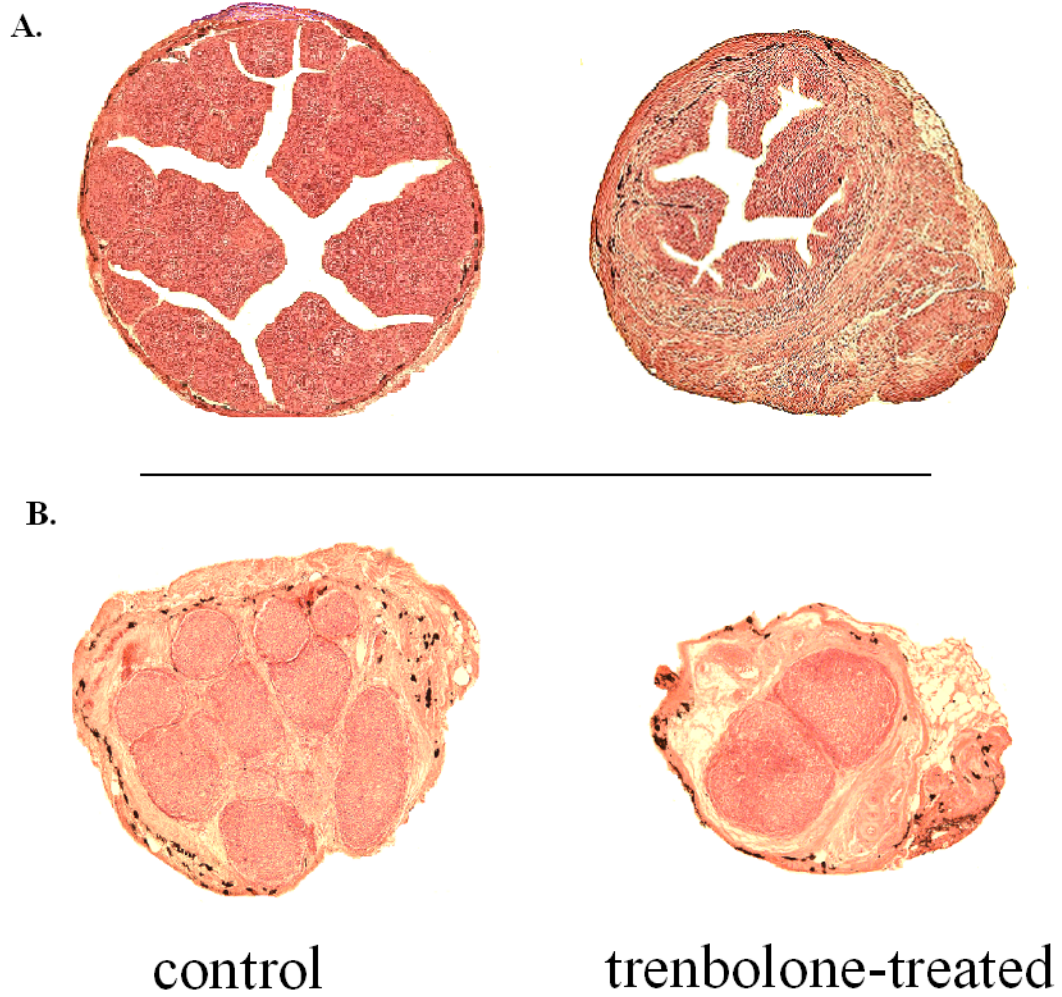
Figure 6. Photographs illustrating the effects of embryonic exposure to trenbolone acetate on the development of follicles and epithelium in bursas of Fabricius from Japanese quail. Bursas collected from one day old quail (A) tended to have smaller and fewer follicles per section. The epithelium surrounding the folds, or plicas, was most often more than twice as thick as controls. Follicle number was also reduced in trenbolone-treated bursas collected from adult quail (B).

Figure 7. Size of follicles (mean +/- SEM) collected from one day old Japanese quail. Significant differences between treatments are indicated by letters (p<0.05).

smaller than controls (p<0.05). Although the follicles from trenbolone-treated adult quail

appeared to be larger than controls, differences in follicle size were not significant.

Although there was some variation in the number of follicles per section from hatchling

bursas from 0.05 to 5 μg trenbolone (Fig 8), there were only about one forth of

the number of follicles from the 50 μg treatment group than controls (p<0.01). These

significant reductions in follicle number remained throughout adulthood. There were less

than half of the number of follicles found in bursas from the 5 and 50 μg treatment levels

than found in controls (p<0.001).

Total leukocyte counts in the 0.5, 5, and 50 μg treatments were approximately

half as large as controls (Fig 9; p<0.05). The heterophil:lymphocyte ratio in chicks from

the 50 μg treatment was twice as large as those from all other treatments (Fig 10;

p<0.05). No significant differences were observed in adult total or differential leukocyte

counts. Hatchling serum IgG levels were significantly elevated in the 0.05 and 0.5 μg

treatment groups (p<0.05), but returned to levels similar to controls at 5 and 50 μg (Fig

11). No significant differences were observed in either of the two humoral response tests

nor the two cell mediated response tests.

DISCUSSION

The teratology studies conducted by industrial laboratories have reported

trenbolone acetate to be "nonteratogenic" (IPCS and CCOHS, www.inchem.org/

documents/jecfa/jecmono/v25je08.htm; 2005), however the current study has shown

embryonic exposure to trenbolone acetate to cause abnormal development of the bursa of

Fabricius in Japanese quail. As seen in studies using exogenous androgens

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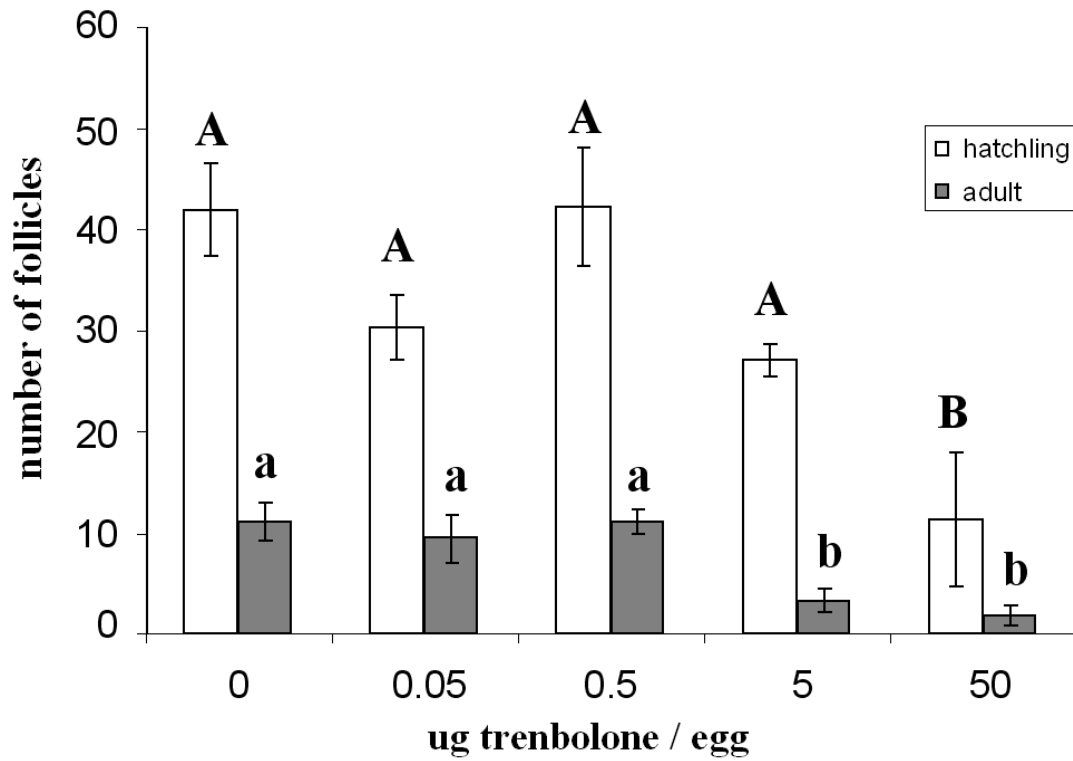
Figure 8. Number of follicles per section (mean +/- SEM) collected from one day old (white bars) and adult (dark bars) Japanese quail. Significant differences between treatments are indicated by uppercase letters for hatchlings and lowercase letters for adults (p<0.05).
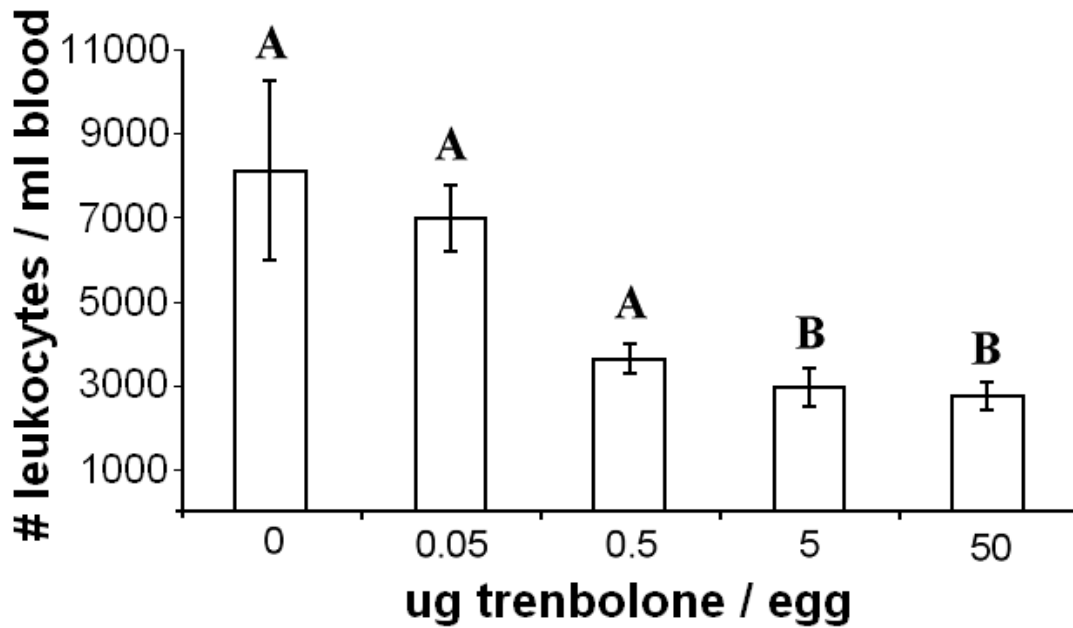
Figure 9. Number of leukocytes per ml blood (mean +/- SEM) collected from one day old Japanese quail. Data was averaged from observations made with two blood smear slides. Significant differences between treatments are indicated by letters (p<0.05).
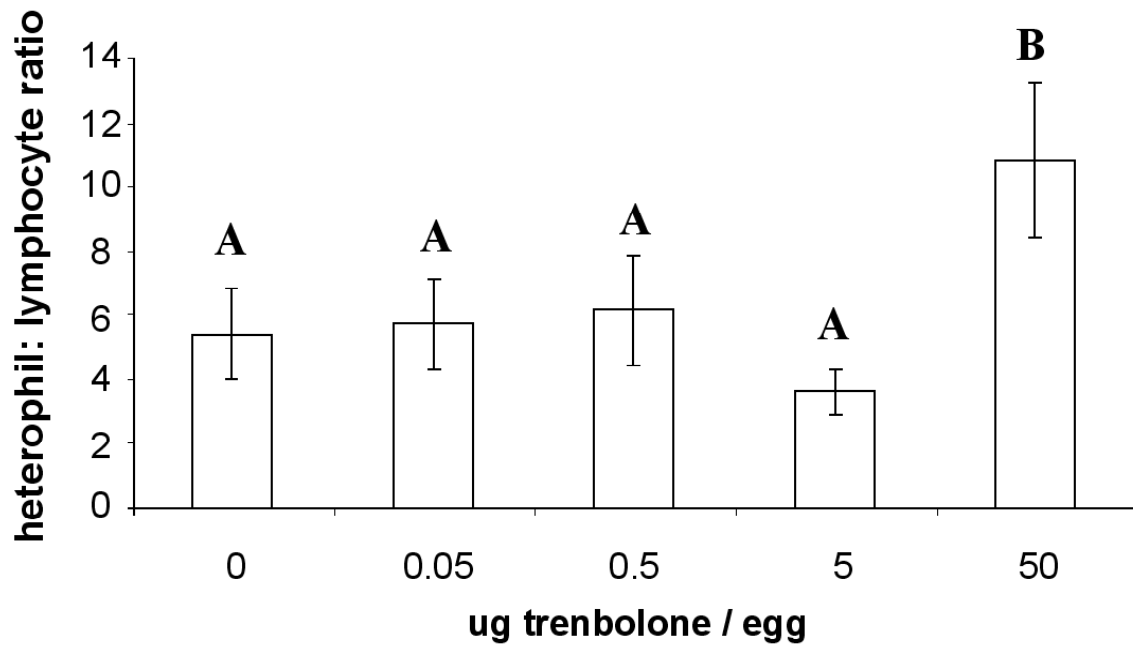
Figure 10. Heterophil:lymphocyte ratio (mean +/- SEM) measured using blood slides made from blood collected from one day old Japanese quail. Significant differences between treatments are indicated by letters (p<0.05).

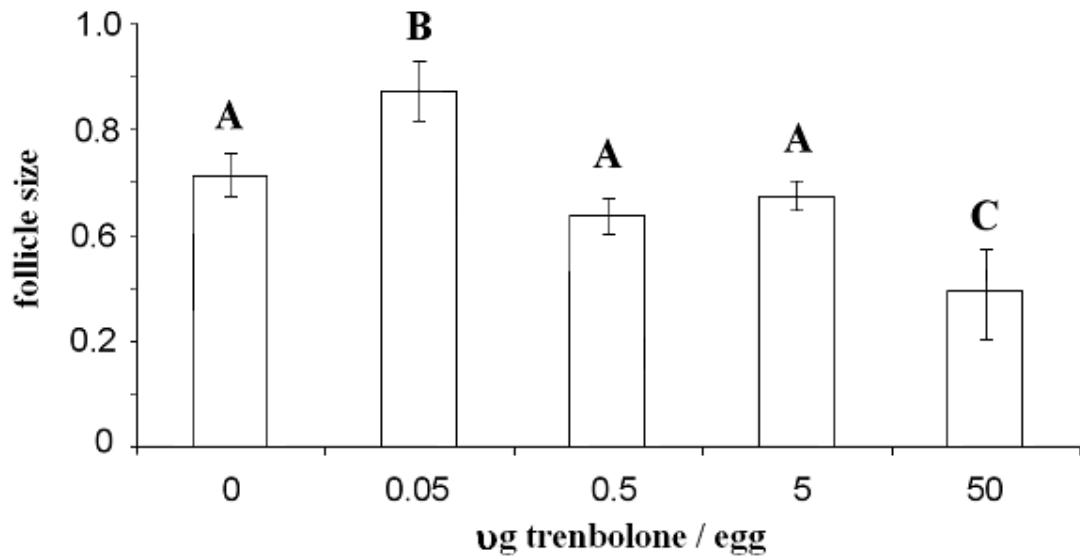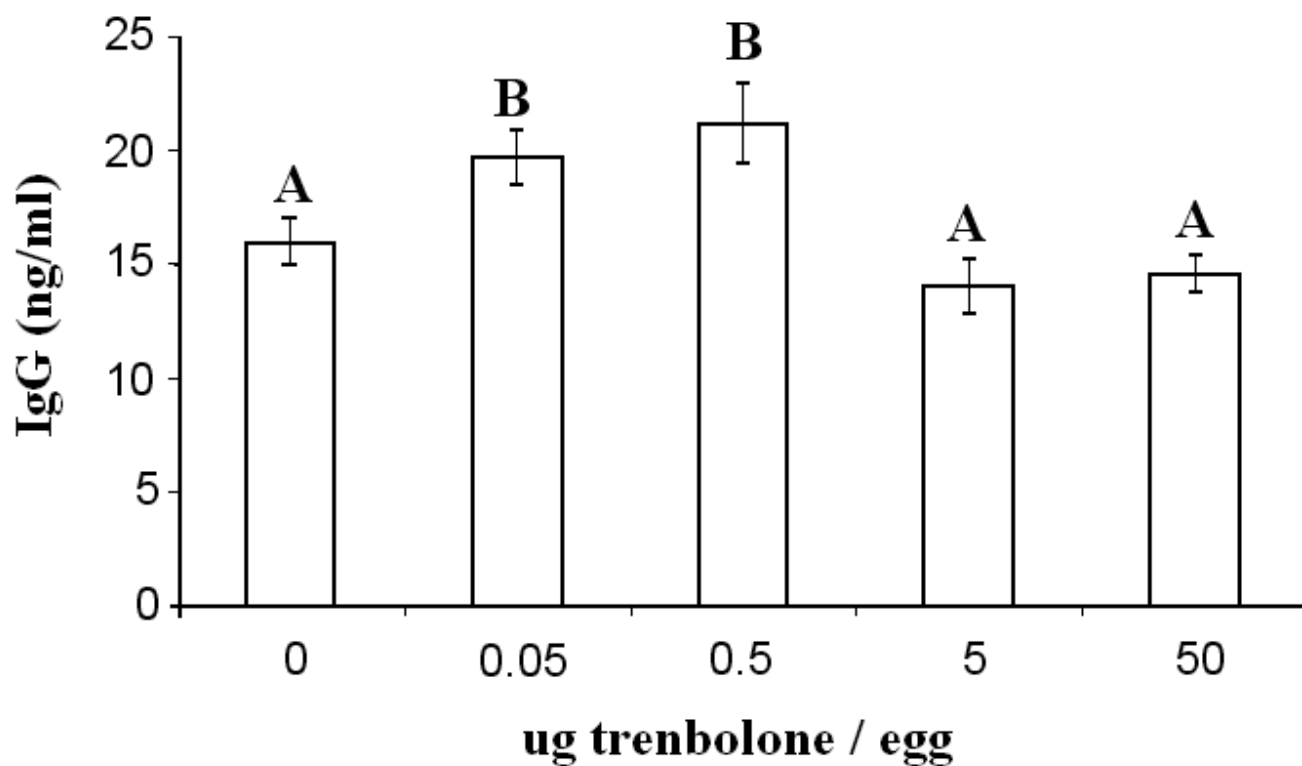Figure 11. Serum immunoglobulin G (IgG) concentrations (mean +/- SEM) collected from one day old Japanese quail. Significant differences between treatments are indicated by letters ($p < 0.05$).

(Glick, 1983; Al-Afaleq and Homeida, 1998), trenbolone caused significant reductions in bursa but not spleen size. Morphological observations revealed that the bursas grown under trenbolone treatment were very similar to those that developed in Japanese quail given testosterone implants at 4 weeks of age (Mase and Oishi, 1991). Formation of the folds, or plicas, was reduced and sometimes non-existent in many of the trenbolone-treated bursas. Also, the fewer follicles that were able to develop under trenbolone treatments exhibited vacuoles, although not to the extent as described by Mase and Oishi (1991). Alterations in follicular development are of great concern since these are the areas of the bursa responsible for B-cell maturation. The differences that were observed in the appearance of the epithelium is also important to note since alterations in this part of the bursa have been known to cause inhibitory effects on the ability of embryonic stem cells to penetrate the bursa from their spleenic migration (Olah et al., 1986). It should be stated, however, that prebursal stem cells seed bursal follicles much earlier than these differences were detected; it is not known if similar changes were occurring at this time.

After observing the ability of adult Japanese quail bursas to recover from significant morphological alterations caused by a similar one-time embryonic exposure to DDE (Quinn et al., IN REVIEW), an anti-androgenic endocrine disrupting chemical, we were surprised to see significant differences in the adult bursas that were collected from the birds in this study that were exposed to a one-time embryonic injection of trenbolone. In many birds, the bursa of Fabricius naturally regresses into a non-functional remnant organ (Pasanen et al., 1998). In Japanese quail, however, the bursa remains throughout adulthood, possibly serving as a secondary lymphoid organ. Therefore, significant

differences observed in the appearance of the adult bursa may indicate altered ability of this organ to function normally.

The heterophil:lymphocyte ratio is a marker of the balance between the nonspecific defenses of heterophils and the antigen-specific defenses of lymphocytes (Shini, 2003). The enhanced heterophil:lymphocyte ratio, therefore, suggests that either antigen specific immune responses may be reduced, or innate responses may be enhanced. Although we did not quantify numbers of B lymphocytes in day old chicks, from our histological observations and from studies that examined bursal B cell maturation under androgen treatments, it is reasonable to suggest that B cell maturation may have been impaired in chicks that received the higher treatment levels of trenbolone.

At the lower treatment levels however, we observed initial increases in the amount of day old plasma IgG. As such, it appears that isotype switch was not inhibited at the lower levels of exposure to trenbolone. Isotype switch may have been inhibited, however, at the 5 and 50 μg treatment levels of trenbolone, as levels of plasma IgG decreased at these doses. Higher levels of IgG could have resulted from either greater numbers of B lymphocytes producing amounts of antibodies similar to controls, or from smaller numbers of B lymphocytes producing significantly greater amounts of antibodies than controls. The decreases in plasma IgG at the two highest treatment levels may have been caused by inhibition of B cell maturation, as suggested by smaller bursas, lower levels of circulating leukocytes, and higher heterophil:lymphocyte ratios (higher ratios possibly being caused by decreased numbers of functional B lymphocytes). In addition, declines in IgG could also be attributed to decreased maturation or increased apoptosis of B cells. It should also be noted that the antibody that was used in the ELISA for the

quantification of serum levels of IgG bound to the constant region of the immunoglobulin molecule. As such, only relative IgG numbers were measured in this study; antibody quality or specificity was not assessed.

Despite the alterations in the structure of the bursa and lymphoid cell population, no significant differences were observed in the CRBC humoral response test or the PHA wing web cell mediated response test. If the increased heterophil:lymphocyte ratios were caused by significant reductions in lymphocytes, one would expect one or both of these tests of antigen specific immune responses to be altered. However, previous studies have shown Japanese quail immune systems to be very resilient to a one-time exposure to androgen active endocrine disrupting chemicals (Quinn et al., IN REVIEW). Also, the immune challenges used in this study were given to the quail as juveniles or adults. Perhaps we would have observed significant differences in immune responses if the challenges were given to the quail as chicks, when the immune system has not yet had a chance to recover from the effects of trenbolone. At this age, immunocompetence is further reduced by the immature status of the immune system. A better test of immunocompetence for Japanese quail embryonically exposed to an endocrine disrupting chemical, therefore, may be one given to individuals soon after hatch.

This study has shown trenbolone to be teratogenic to the development of the bursa of Fabricius in Japanese quail. Since development of a healthy avian immune system is dependent on normal growth and development of the bursa, disruption of bursal development may impact individual survival and have subsequent effects on a population level. More studies are needed to further assess the potential of trenbolone as an endocrine disrupting chemical and to determine exposure levels in wild bird populations.

Chapter 4: Effects of DDE on the Reproductive System

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are environmental contaminants that act as hormone agonists or antagonists. As such, these chemicals have the potential to alter the development and function of all systems that are influenced by the endocrine system. The effects of these chemicals on the reproductive system have traditionally been of great concern due to the structural similarities that many share with sex steroids. Also, there is a growing literature, that demonstrates the ability of these chemicals to disrupt reproductive function, thereby creating potential negative effects on individual fitness and population dynamics (Fry, 1995). Many EDC-induced effects on reproduction, growth, behavior, and declines in wild populations have been reported for birds over the past three decades (Fox et al., 1978; Kubiak et al., 1983; Becker et al., 1993). What remains ambiguous, however, are the underlying mechanisms for these effects.

A sexually dimorphic pattern of gonadal steroids modulates the sexual differentiation of accessory sex structures and neuroendocrine systems that regulate endocrine and behavioral components of reproduction (reviewed in Ottinger et al., 2001). This differentiation of the hypothalamic component of the hypothalamic-pituitary-gonadal (HPG) axis occurs mainly during late embryonic and early posthatch development (Ottinger 1989). Competition between endogenous hormones and EDCs could alter the relative exposure to androgens and estradiol, thus altering normal development of both the endocrine and behavioral components of reproduction. Steroid hormones also activate many sex-specific endocrine and behavioral responses during

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puberty. The success of this activation, however, relies on appropriate organization of these neural systems during embryonic development. If development of these responses was altered by embryonic exposure to EDCs, the likelihood of proper activation at puberty and adulthood is reduced.

DDE [ethylene, 1,1-dichloro-2,2-bis(p-chlorophenyl)] is a an anti-androgenic EDC that acts as a potent androgen receptor antagonist (Kelce et al., 1995). DDE is the primary metabolite of the pesticide DDT [1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane], which was used widely as a potent contact poison against arthropods from the 1940s until its ban in most major industrial countries in the 1970s. Since its ban, levels of DDT have been steadily declining, however DDE still remains relatively high in the world's environmental and organismal systems, as it is chemically more stable and biologically more persistent than its parent compound. DDE is an effective testosterone hydroxylase modulator as well as an inducer of hepatic aromatase (You, 2000).   Increased activity of these enzymes reduce circulating testosterone through its conversion to either androstenedione, a less potent androgen, or 17β–estradiol.

Many observational studies have examined potential associations between DDE exposure and reproductive effects with varying results (Nygard and Gjershaug, 2001; Gill et al., 2003; Rattner et al., 2004; Reynolds et al., 2004). One of the major limitations of observational studies is that exposure to other EDCs cannot be controlled for, precluding any possibilities of determining causality. Many studies have focused on DDE's effects on eggshell thinning (Hazeltine, 1972; Bowerman et al., 1995; Blus et al., 1997), paying less attention to more subtle reproductive effects such as behavior and maturation. Finally, many of the laboratory studies conducted to determine the reproductive effects of

DDE in birds used dietary treatments. Although dietary studies are crucial for the complete understanding of the effects of a chemical on environmentally relevant reproductive measures, only post-hatch exposure occurs. Since DDE is lipophilic, maternal deposition into the yolk is the primary route of exposure for embryos, which impacts development of the reproductive system. Assessment of overall reproductive effects must consider embryonic exposure.

The developing embryo often becomes exposed to a myriad of EDCs during one of the most sensitive stages of development. Furthermore, many of the developing endocrine systems are vulnerable to environmentally relevant levels of EDCs at different stages in ontogeny, and the resulting impact may be subtle. Therefore, traditional toxicological measures are not always sufficient to measure long-term, chronic effects that lead to population-level impacts. The objective of this study was to examine the effects of an androgen-active EDC on reproductive developmental and behavioral responses in Japanese quail (*Coturnix japonica*). Japanese quail was chosen as a model species in this study because they are well suited for toxicity tests (Fair et al., 1999). Japanese quail are particularly useful in studies that examine bursal development and function because unlike most avian species, the quail maintains a bursa throughout life (Pardue, 1981). Exposure to DDE was hypothesized to disrupt sexual differentiation of the gonads and brain, as measured by onset of puberty, gonad size and morphology, and adult male reproductive behavior. The data produced by this study provide a more realistic view of the capacity of an avian species to respond to EDC challenges during sensitive phases in development and examine long-term consequences of exposure on the reproductive system critical to the fitness of the individual. Furthermore, this study will

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add to the literature on EDC effects in Japanese quail as as avian model for regulatory toxicity testing.

METHODS

Eggs were collected from the Department of Animal and Avian Sciences Japanese quail (*Coturnix japonica*) colony that is a random bred white egg producing strain. The colony is maintained under controlled light (15L:9D), temperature, and humidity according to Institutional Animal Care and Use Committee (IACUC) approved standard operating procedures. Feed (Purina Game Bird Layena, Purina, St. Louis, MO) and water were available *ad libitum*. This strain of Japanese quail are relatively small (100-130 gm), and reproductive function is entirely regulated by long photoperiod.

One hundred eggs per treatment were randomly assigned to either control (sesame oil) low DDE (20 μg/egg) or high DDE (40 μg/egg) treatments. Treatments were injected into the yolk (20 μl) to mimic maternal deposition of the EDCs. The low dose was selected to reflect environmentally relevant levels that should not affect reproduction, but may affect bursal development, whereas the high dose was also environmentally relevant at a level previously found to impact reproduction (Henny et al., 1984; Custer et al., 1983; Custer and Mitchell, 1987; White et al., 1988; Henny and Herron, 1989; Nisbet, 1989). Holes at the injection site were made with a 20-gauge needle at the apex of the egg after being cleansed of fecal material and blood with 70% ethanol. The injection sites were immediately sealed with melted paraffin, and the eggs were immediately set to incubate.

After hatch, quail were allowed to dry in the incubator and then placed into heated brooders (95°F, 24 h light; temperature was reduced 5°F every week) until four weeks of age. Diet and water were provided *ad libitum* (Purina Gamebird Startina, Purina, St. Louis, MO, USA). At four weeks, quail were separated and housed in individual cages at room temperature and on a long-day photoperiod (16:8 light:dark) so onset of puberty could be monitored in individuals. Chicks were switched to adult diet (Purina Gamebird Layena, Purina, St. Louis, MO, USA), and feed and water were provided *ad libitum*.

Onset of puberty was assessed in females as the first day of egg production. In males, onset of puberty was measured as the first day of foam production from the proctodeal gland. The proctodeal, or cloacal, gland is an androgen-sensitive secondary sex character in Japanese quail and an external index of sexual maturity in the male (Watson and Adkins-Regan, 1989; Mohan et al., 2002). Cloacal gland area (length X width) was determined at eight weeks of age, a time that 90% of the control quail were sexually mature to serve as a comparison of status of maturation across the treatments.

Male copulatory behavior was also assessed at 8 weeks of age, to assess sexual maturation and behavioral responses. Males were individually housed at 4 weeks of age and each male was tested on three consecutive days. In each test, male behavior was observed for three minutes following introduction of a sexually receptive female into the male's cage. Females were similarly aged and taken from the colony; a female was replaced if she was aggressive or unreceptive. Time of latency to mount, the number of mount attempts, and the number of successful cloacal contacts were recorded.

After the reproductive behavior tests were completed, males and females were paired. Birds were given one week to adjust, and then at 10 weeks of age, egg production

was measured as the average number of eggs produced per individual in one week. Eggs were collected over the following week and stored at 7°C, incubated, and examined at 14 days incubation to determine fertility and development. Percent fertility was calculated as the average number of eggs that were fertililized, and percent viability was calculated as the average number of embryos that were alive when the eggs were opened.

At 12 weeks of age, three eggs were collected per pair and prepared for analysis of sperm penetration of the perivitelline layer as described by Donoghue (1996). Briefly, the perivitelline membrane was excised, mounted on a slide, and stained with Schiff's reagent. The germinal disc was centered in the field of view and all holes caused by sperm digestion were counted at 100x under light microscope. The data were analyzed as the average number of holes observed in the three samples per pair.

All adult birds were sacrificed at 18 weeks of age. Combined testes weights were expressed as relative to body weight. The left testes was fixed in Bouin's solution immediately after collection. Testes were washed three times in 50% ethanol (6 h each), dehydrated through a series of increasing concentrations of alcohol, and infiltrated and embedded in paraffin blocks. The blocks were sectioned at 10μm thickness on a rotary microtome and mounted on slides. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin according to Addison (1929). The relative area occupied by spermatazoa within a seminiferous tubule was measured, and spermatogenic stages were checked to verify normal spermatozoan maturation. The area within the testis occupied by sperm cells was determined using image analyses (IP Lab 3.6 for Windows, Scanalytics Inc., Fairfax, VA, USA). The area occupied by cells within a seminiferous

tubule was also determined, which would include both spermatozoa and Sertoli cells. Five randomly selected seminiferous tubules were measured and averaged for each bird.

Immediately after the dissection of the left epididymis, a drop of epididymal fluid containing spermatozoa was collected from the end of the caudal portion of the epididymis. The sample was placed on a prewarmed slide (37 °C) with two drops of phosphate-buffered saline and was coverslipped. Slides were analyzed under ×400 by two independent observers, who examined five separate fields for each sample/observer. The percentage of sperm that was motile was estimated. A spermatozooa was considered motile if it showed forward movement in a progressive consistent path. Motility was estimated to the nearest 10% for each sample, and the two ratings were averaged per sample. Ovary weights relative to body mass and the number of mature yellow follicles were measured in females.

The Statistical Analysis System (SAS Institute, Inc. 1987) was used for all statistical analyses. Assumptions for parametric statistics were examined prior to analysis. Data were analyzed separately by sex when sex-by-treatment interactions were significant. All measurements were analyzed by two-way analysis of variance, and Tukey tests were used for post hoc pairwise comparisons.

RESULTS

Onset of puberty was accelerated in females from the 20μg treatment group by approximately one week compared to controls (Fig. 12; $p < 0.05$), as measured by date of first egg laid. No differences in onset of puberty in males or cloacal gland size were observed among treatments.
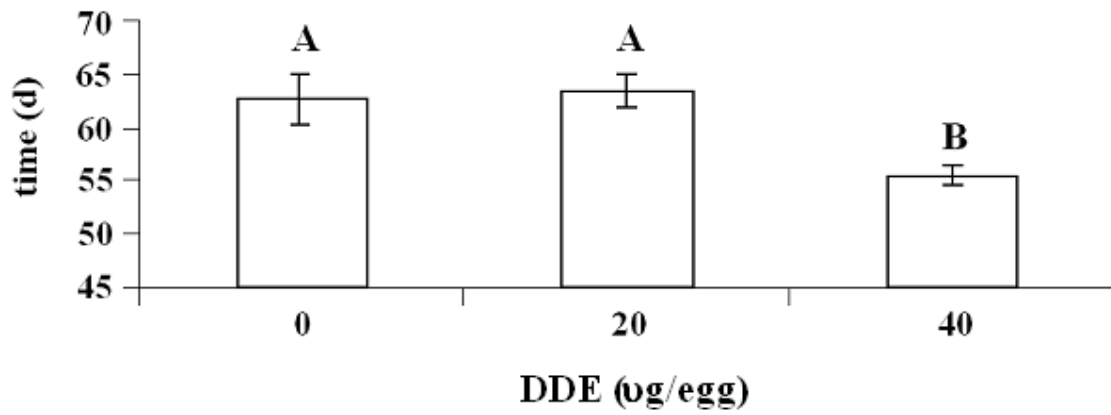
Figure 12. Day of onset of puberty in females (mean +/- SEM) measured as first day of egg laid in Japanese quail. Significant differences between treatments are indicated by letters (p<0.05).

Significant differences in copulatory behavior were observed only on the first day of testing. During the first day's test, males from the 20 and 40 µg treatment groups attempted to mount females about only 75% the number of times as controls (Fig. 13; $p<0.05$). Also, males from the 40 µg treatment group took approximately twice as long to achieve a successful copulation as control quail (Fig 14; $p< 0.05$). Although it took males from the high treatment level of DDE longer to achieve successful cloacal contacts, the resulting number of successful contacts that were made within the test's limit of three minutes did not differ among treatments.

Differences in the area of seminiferous tubules approached significance ($p = 0.07$); seminiferous tubules in testes from quail of the 40 µg treatment group were 15% larger than controls. No other morphological differences were observed; testes weight and sperm motility did not differ among treatments. Also, no significant differences were observed among females with ovary weights and numbers of mature yellow follicles.

DISCUSSION

Recent studies have reported increasing avian populations in traditionally highly polluted areas following the ban of DDT in the early 1970s (Rattner et al., 2004 and 2000; Fasola et al., 1987; Spitzer et al., 1978). Many previously threatened populations have also begun to even attain numbers comparable to dates preceding heavy use of organochlorines. This increase in bird numbers correlates with a decrease in DDE residues found in eggs and individuals; the levels of contamination found in post-ban populations often have been below the critical threshold for reproductive effects (Rattner et al., 2000; Fasola et al., 1987). Specific effects, such as eggshell thinning and

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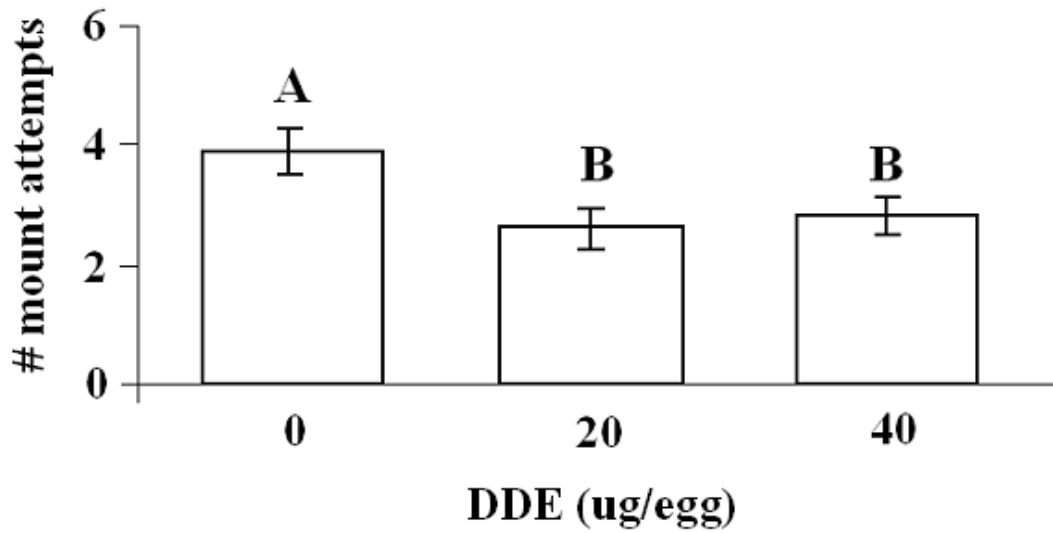
Figure 13. Number of times male Japanese quail attempted to mount females (mean +/-SEM) in the first of three reproductive behavioral test trials. Significant differences between treatments are indicated by letters (p<0.05).
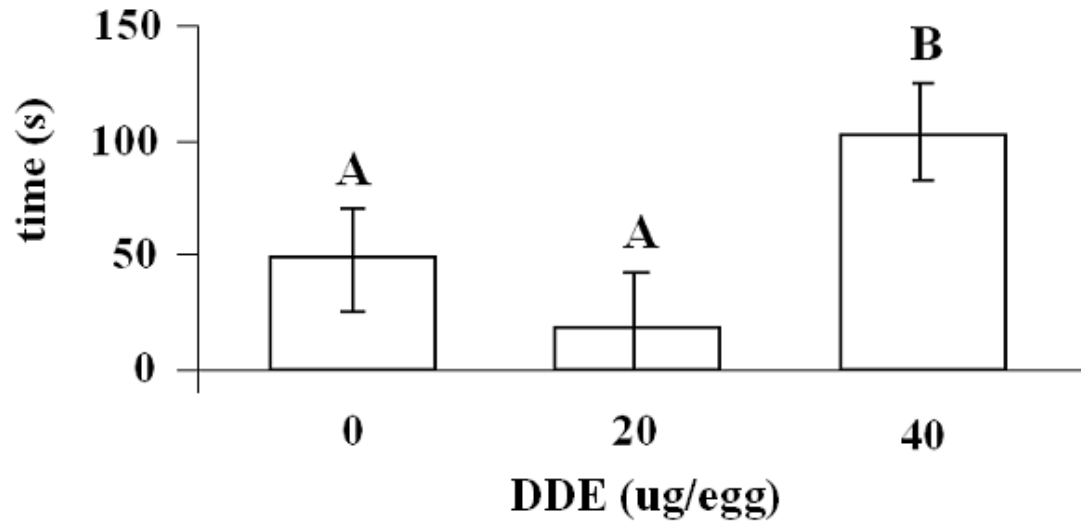
Figure 14. Amount of time to achieve successful copulations (mean +/- SEM) during the first of three reproductive behavioral test trials by adult male Japanese quail. Significant differences between treatments are indicated by letters (p<0.05).

reproductive output, have also been restored as DDE contamination decreases, leading

many to conclude that the current impacts of DDE in certain populations are negligible.

Although encouraging, one should be cautious in using this data to make predictions for

all wild bird populations. Even in those populations where numbers of individuals are on

the rise, the potential exists that these are still insufficient enough to sustain some

populations.

Other studies have shown that DDE is still prevalent in many food webs,

suggesting that DDE contamination is still a significant factor in wild populations (Custer

et al., 2001). Our study demonstrates effects of DDE on the onset of puberty in females.

This effect may have positive as well as potentially negative impacts on the reproductive

performance of the individual. Data in domestic poultry have shown that early

stimulation of egg laying by photostimulating young hens impacts egg weight and causes

reductions in chick weight (Siopes, 1992; Joseph et al., 2002). Conversely, acceleration

of the onset of reproductive ability may create the potential for contaminated individuals

to reproduce earlier and gain a reproductive advantage. This could have far reaching

effects at the population level if less fit genes become contributed to future generations.

Also, although no significant differences were observed with gonadal physiology or

morphology, reproductive behavior was inhibited. Clearly, an individual cannot

contribute to the fitness of a population if they show no sexual behavior, even if gonadal

function is completely functional.

Contamination levels of DDE still remain high in some avian populations

(Bartuszevige et al., 2002; Nygard and Gjershaug, 2001), sometimes being the most

prevalent organochlorine compound to be detected. However, the reproductive effects do

not always relate to the detected contamination levels, possibly due to differential species' sensitivity to DDE exposure (Nygard and Gjershaug, 2001; Lundholm, 1997; Longcore et al., 1971). Discrepancies in the effects of DDE may also be due to exposure to different congeners of DDE, as described by Lundholm (1997). Further, birds feeding in varied trophic guilds differ in accumulation of DDE. For example, insectivorous passerines have significantly higher levels of DDE than omnivores and granivores (Bartuszevige et al., 2002). Golden eagles, whose prey consists chiefly of marine birds, have higher levels of DDE than eagles preying on terrestrial herbivores (Nygard and Gjershaug, 2001). These variables must be considered when determining the potential impact of DDE and its congeners on avian species.

Finally, adult birds often appear to adapt to low levels of contaminant exposure, and as such, traditional reproductive measures of toxicity may not be adequate to assess overall effects on fitness. This adaptability was observed in captive black ducks that continued to reproduce while fed DDE-contaminated feed for two years. They showed improved reproductive success and eggshell quality when placed on untreated food for an additional two years (Longcore and Stendell, 1977). Moreover, as mentioned above, DDE impacts other physiological systems, including the immune system. In the current study, these quail chicks exposed to DDE also exhibited severe immune impairment, which disappeared as the birds reached adulthood (Quinn et al., in review). Again, although results such as these are encouraging for the resilience and survival of the adults, at some point effects on the chicks will impact the population as a whole. Japanese quail have been shown to be fairly resistant to DDE-induced reproductive impairment to dietary doses as high as 200 ppm as measured by number of eggs laid, egg

weight, or eggshell thickness (Davison et al., 1976). However, in the current study, low one-time *in ovo* exposure to DDE resulted in significant reductions in copulatory behavior. Although testis morphology and other measures of reproductive function appeared unaffected, these males would not be predicted to have normal fertility due to the decrease in appropriate behavior. This study suggests that the neuroendocrine system may be more sensitive and less resilient to embryonic contaminant exposure than traditional measures of reproductive success, and should be considered more often in toxicity tests.

Chapter 5: Effects of Trenbolone Acetate on the Reproductive System

INTRODUCTION

Many studies have shown sex hormones released from human and animal wastes to have endocrine-disrupting effects, and have most often focused on the effects of natural and synthetic estrogens (Tilton et al., 2002; Metcalfe et al., 2001; Purdom et al., 1994). A lot of attention has been directed towards the synthetic estrogens since they are more environmentally stable and more resistant to microbial degradation than the natural steroids (Tabak et al., 1981; Tabak and Bunch, 1970). Few studies have considered possible endocrine-disrupting activities of androgenic compounds in human and animal excreta, especially residues from agents in the feed of domestic species.

Trenbolone acetate (17β-acetoxyestra-4,9,11-triene-3-one) is a synthetic androgen that is used in many meat-exporting countries. Upon consumption, trenbolone is almost immediately hydrolyzed to its active metabolite trenbolone-17β, which has an affinity to the recombinant human androgen receptor similar to dihydrotestosterone (Bauer et al., 2000). The long half-life of trenbolone and its persistence in soils treated with manure from animals given implants (Schiffer et al., 2001) create the potential for this synthetic androgen to accumulate in soils and in higher trophic levels of food webs. The potential for endocrine disrupting problems is evident when considering that in the US alone, several tons of trenbolone are applied each year (Schiffer et al., 2001).

Although many studies have assessed the potential teratogenic effects of trenbolone, much of these data, collected in industry laboratories, remain unpublished (Wilson et al., 2002). Despite the potential for environmental effects, very few studies

have been done to test trenbolone's effects on wildlife. Teratological studies conducted by industrial laboratories have reported trenbolone to be "nonteratogenic" (IPCS and CCOHS, www.inchem.org/documents/jecfa/jecmono/v25je08.htm; 2005). In separate analyses, we have demonstrated that *in ovo* exposure to trenbolone inhibited proper development and function of the immune system in Japanese quail (Quinn et al., in review). It is, therefore, important to reevaluate the potential of trenbolone to act as an endocrine disrupting chemical (EDC) with teratogenic properties.

During ontogeny, male and female embryos experience a sexually dimorphic pattern of gonadal steroids, which organizes the sexual differentiation of accessory sex structures and neuroendocrine systems that regulate endocrine and behavioral components of reproduction (Ottinger et al., 2001). This differentiation of the hypothalamic-pituitary-gonadal (HPG) axis occurs mainly during embryonic development, with organization of hypothalamic neural systems occurring during late embryonic and early posthatch development (Ottinger 1989). As such, embryonic exposure to chemicals that mimic or alter the signals of hormones can disrupt the natural development of this system and have lasting effects that may persist throughout adulthood.

The purpose of this study was to examine the consequences of embryonic exposure to trenbolone acetate on reproductive development and copulatory behavior in Japanese quail. Therefore, we hypothesized that *in ovo* exposure to trenbolone acetate would disrupt sexual differentiation of the gonads and brain through its androgenic activity, and that the endocrine disruption would be expressed by altered onset of puberty and diminished adult reproductive function. These data, in combination with observed

detrimental effects on the development and function of the immune system (Quinn et al., in review), help document the scope of effects of varied classes of EDCs in birds and help to identify appropriate measurement end points for use in ecological risk assessment.

METHODS

Japanese quail (*Coturnix japonica*) was chosen as a model species in this study because they are well suited for toxicity tests (Fair et al., 1999). Eggs were collected from a colony at the Department of Animal and Avian Sciences at the University of Maryland, College Park. Ninety eggs per treatment were randomly selected to receive either sesame oil (control), 0.05, 0.5, 5 or 50 μg trenbolone acetate (Sigma Chemical Co., St. Louis, MO, USA). Trenbolone was dissolved in sesame oil and injected into yolks at a volume of 20 μl on day four of incubation. Treatments were injected into the yolk to mimic maternal deposition. Levels of trenbolone in yolks in the environment have not been determined. Holes at the injection site were made with a 20-gauge needle at the apex of the egg after being cleansed of fecal material and blood with 70% ethanol. The injection sites were immediately sealed with melted paraffin, and the eggs were immediately set to incubate.

After hatch, quail were placed into heated brooders (95°C, 24 h light) until four weeks of age. Diet and water were provided *ad libitum* (Purina Gamebird Startina, St. Louis, MO, USA). At four weeks, quail were separated and housed in individual cages at room temperature and on a long-day photoperiod (16:8 light:dark) so onset of puberty could be monitored in individuals. Diet and water were provided *ad libitum* (Purina Gamebird Layena, St. Louis, MO, USA).

Onset of puberty was assessed in females as the first day of egg production. In males, onset of puberty was measured as the first day of foam production from the proctodeal gland. The proctodeal, or cloacal, gland is an androgen-sensitive secondary sexual characteristic in Japanese quail (Watson and Adkins-Regan, 1989; Mohan et al., 2002). At approximately eight weeks of age, after 90% of the quail had been sexually mature for two weeks or longer, the area of the proctodeal gland was measured.

Male copulatory behavior was also assessed at 8 weeks of age. These tests were conducted over three consecutive days for each male. For each test, behavior was observed for three minutes as soon as a sexually receptive female was introduced into the male's cage. Females were replaced if found to be aggressive or unreceptive. Time of latency to mount, the number of mount attempts, and the number of successful cloacal contacts were recorded.

After the reproductive behavior tests were completed, males and females were paired by similar weights. At 10 weeks of age, egg production was measured as the average number of eggs produced per individual in one week, after which, eggs were collected over the following week and stored at 7°C. Eggs were then incubated and opened after two weeks. Percent fertility was calculated as the average number of eggs that were fertililized, and percent viability was calculated as the average number of embryos that were alive when the eggs were opened.

At 12 weeks of age, three eggs were collected per pair and prepared for analysis of sperm penetration of the perivitelline layer as described by Donoghue (1996). When the slides were ready for quantification of the holes caused by sperm digestion, the slides were observed at 100x under a light microscope. The germinal disc was centered in the

field of view and all holes in this field were counted. Data were analyzed as the average number of holes observed in the three samples per pair.

All adult birds were sacrificed at 18 weeks of age. Testes weights relative to body size were analyzed in males. Immediately after the dissection of the left epididymis, a drop of epididymal fluid containing spermatozoa was collected from the end of the caudal portion of the epididymis. The sample was placed on a prewarmed slide (37 °C) with two drops of phosphate-buffered saline and was coverslipped. Slides were analyzed under 400× by two independent observers, who examined five separate fields for each sample/observer. The percentage of sperm that was motile was determined. A spermatozooa was considered motile if it showed forward movement in a progressive consistent path. Motility was estimated to the nearest 10% for each sample, and the two ratings were averaged per sample. Ovary weights relative to body mass and the number of mature yellow follicles were measured in females.

The Statistical Analysis System (SAS Institute, Inc. 1987) was used for all statistical analyses. Assumptions for parametric statistics were examined prior to analysis. Data were analyzed separately by sex when sex-by-treatment interactions were significant. All measurements were analyzed by two-way analysis of variance, and Tukey tests were used for post hoc pairwise comparisons.

RESULTS

Onset of puberty was delayed in males from the 50 μg treatment group by approximately one month longer than controls (Fig.15; $p < 0.001$), as measured by initial date of foam production by the cloacal gland. Adult cloacal gland area (Fig. 16) was
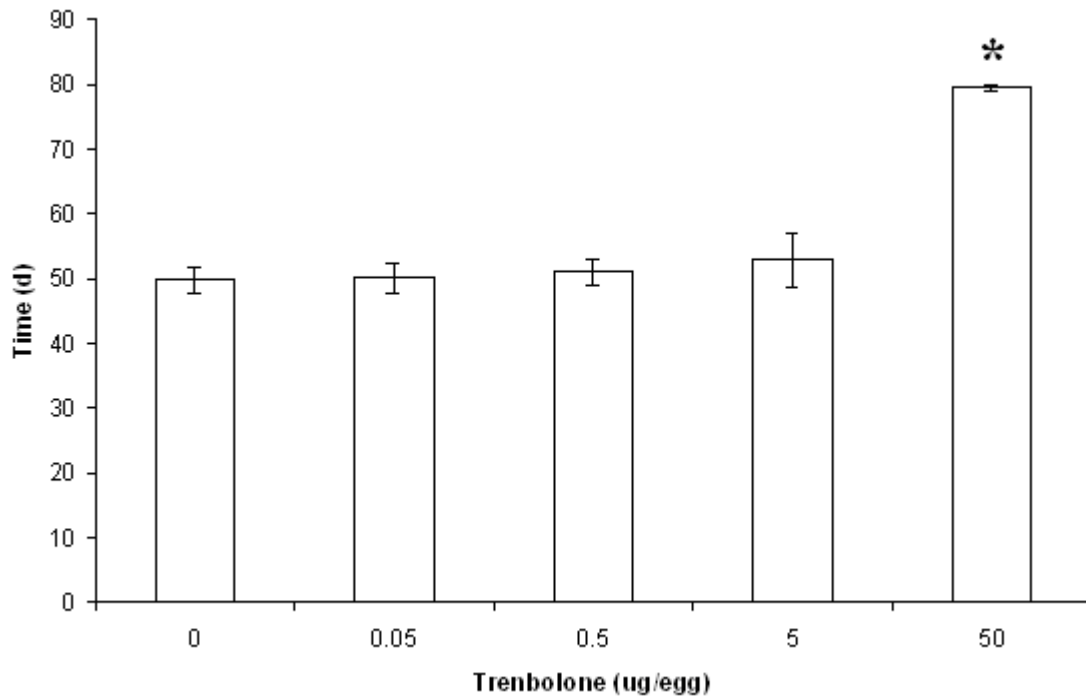
Figure 15. Day of onset of puberty in males (mean +/- SEM) measured as first day of foam production by the proctodeal gland in Japanese quail. Significant differences between treatments are indicated by asterisk ($p < 0.001$).
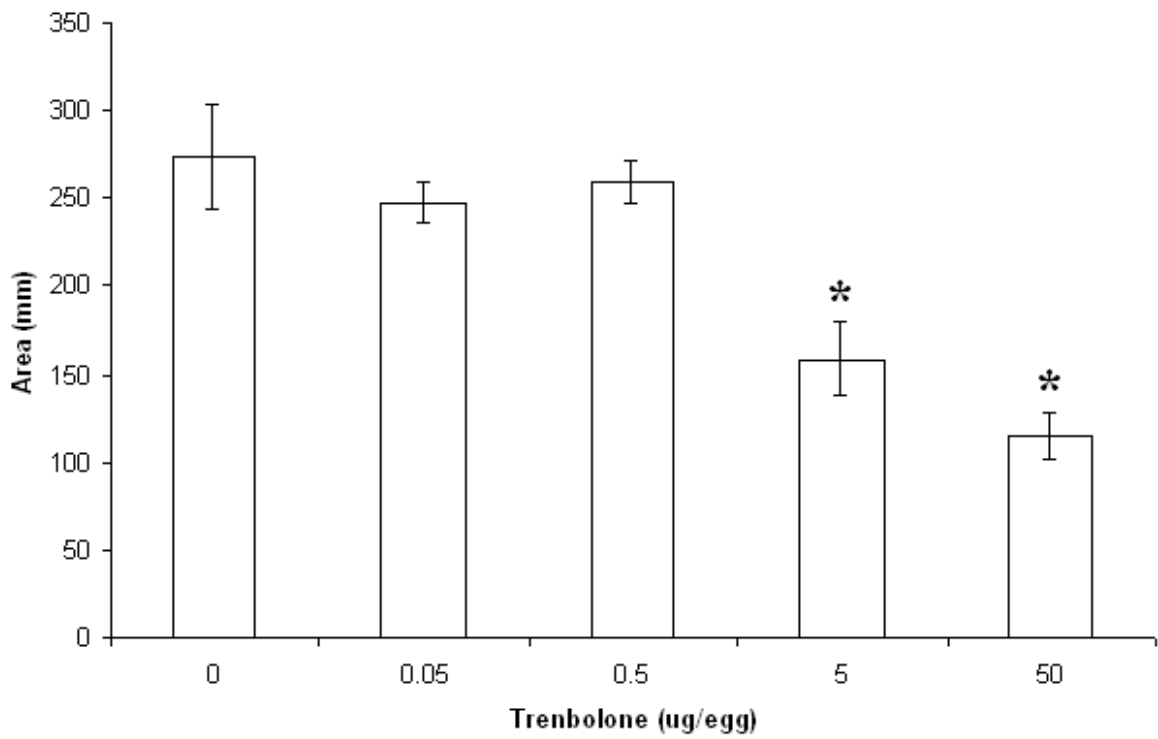
Figure 16. Size of proctodeal gland (mean +/- SEM) in adult Japanese quail. Significant differences between treatments are indicated by asterisk (p<0.0001).

approximately 40% smaller than controls in the 5 μg treatment group and about half as large as controls in the 50 μg treatment group (p < 0.0001). No differences in onset of puberty were observed in females, as measured by date of first egg laid. No differences in gonadal physiology or morphology were observed in either sex.

Significant differences in copulatory behavior were found in the first of the three days of testing suggesting an effect of experience. Adult males from the 50 μg treatment group attempted to mount females (Fig. 17) less than one-third the number of times as controls (p<0.05). The number of mount attempts observed in quail from the 0.05, 0.5, and 5 μg treatment groups did not differ significantly from controls or the 50 μg treatment group, however the number of attempts in this middle range was approximately one-half of that observed in controls. The number of successful copulations achieved by males was significantly reduced in all treatment levels compared to those from the control treatment (Fig. 18; p < 0.01). No differences among treatments were observed with latency to mount.

DISCUSSION

An individual's fitness is its relative contribution to the gene pool of future generations. Therefore, fitness contributes to a population's survivorship, which is benefited by having the heartiest individuals reproduce. If exposure to trenbolone delays the onset of puberty in males, it is possible that less fit individuals who are not exposed to the chemical might get a reproductive advantage over more fit ones who are. In this study, puberty was delayed by about a month in the highest treatment level. If less fit males were able to reproduce before more fit males, population effects could result where

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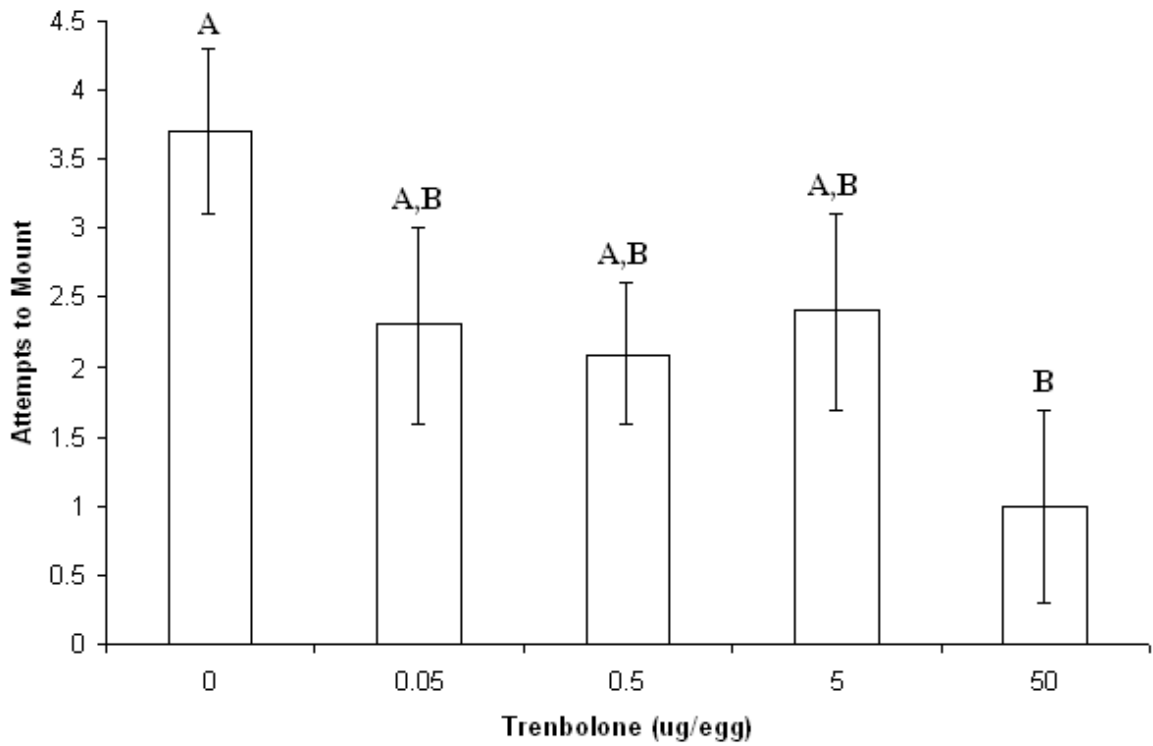
Figure 17. Number of times male Japanese quail attempted to mount females (mean +/- SEM) in the first of three reproductive behavioral test trials. Significant differences between treatments are indicated by asterisk (p<0.05).
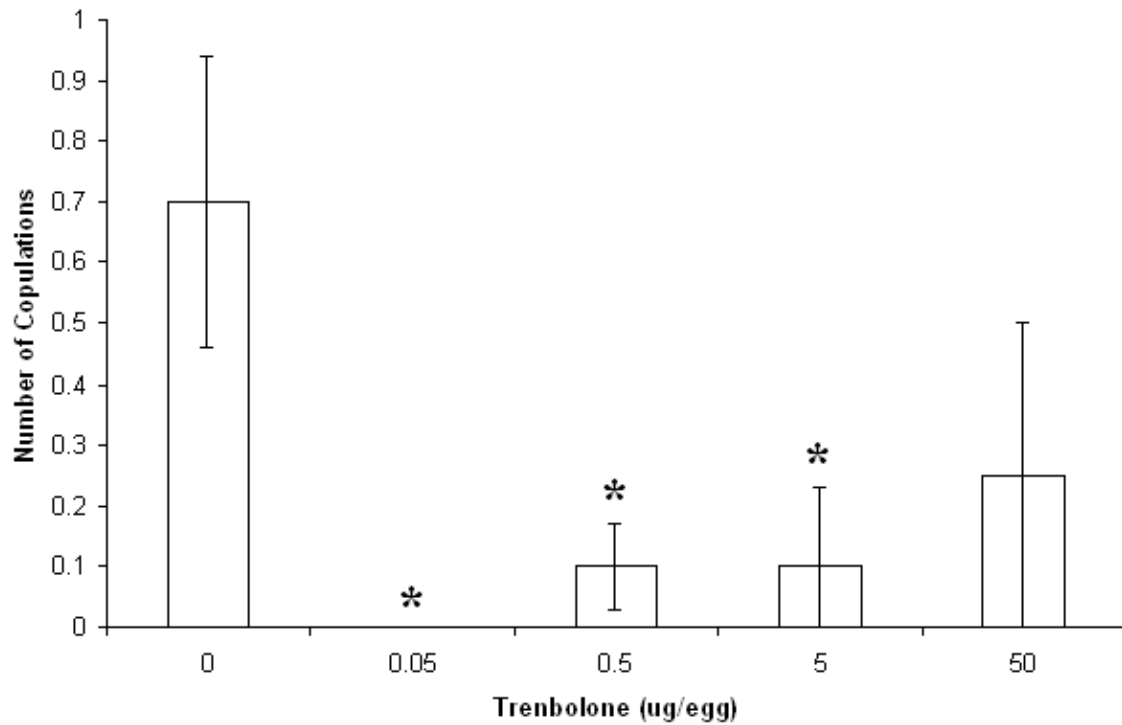
Figure 18. Number of successful copulations (mean +/- SEM) achieved during the first of three reproductive behavioral test trials by adult male Japanese quail. Significant differences between treatments are indicated by asterisk (p<0.01).

less fit genes become added to the genotype of the population rather than ones that might contribute strength, greater immunocompetence, and fertility.

Onset of puberty was measured by the time at which the cloacal gland first became functional. Cloacal gland area is often used as an indicator of male reproductive development, as its growth and function are dependent on circulating levels of testosterone (Ottinger and Brinkley, 1978). The hypothalamic-pituitary-gonadal axis (HPG axis) begins to become sexually dimorphic in late embryonic development in Japanese quail, during what is termed the "critical period" that later determines male or female endocrine and behavioral patterns (Ottinger et al., 2001; Ottinger, 1989). Disruption of the formation of the HPG axis would likely affect its later activation, which could impact the onset of puberty. The feedback system between gonadotropin-releasing hormone (GnRH) and gonadal steroids is initiated by embryonic day (ED) 10. Changes in the relative ratio of the concentration of testosterone to that of estradiol play more of a role in sexual differentiation of the HPG axis than absolute amounts of either individually (Ottinger 1989). This ratio could have been altered by exposure to androgenic trenbolone, thereby altering the feedback system between GnRH and the gonadal steroids.

The neuroendocrine system may be one of the most sensitive areas to be affected by EDCs during embryonic development, and alterations to its development could have long lasting effects on behavior exhibited throughout adulthood. Specifically, in this study, our data suggests that the neuroendocrine system responsible for the development and interaction of the reproductive system may be more sensitive to embryonic exposure to trenbolone acetate than the gonads. No differences in male or female gonadal function

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were detected, however, reproductive behavior was inhibited. The use of behavioral

measurements as biomarkers of exposure to EDCs often gets overlooked in reviews of

appropriate and sensitive EDC-exposure endpoints (Fossi et al., 1999). Tests that solely

examine the effects of EDCs on the morphology and functioning of organ systems may

not be adequate enough to determine the complete toxicity of a compound. Intact and

functional gonads do not contribute to an individual's fitness if the performance of

reproductive behavior is impaired. Behavior should, therefore, be included in traditional

toxicity tests, as it is not only a non-lethal method to measure alterations in brain

development, but also provides a method of linking physiological function with

ecological processes (Scott and Sloman, 2004).

Copulatory behavior in male Japanese quail is controlled by the sexually

dimorphic medial preoptic area (POA) or nucleus (Balthazart et al., 2000). Many studies

have elucidated the early embryonic changes in the anatomy and neurochemistry of the

POA that set into place the mechanisms behind the activation of adult reproductive

behavior (reviewed in Castagna et al., 1999). Male Japanese quail copulatory behavior

has been experimentally demasculinized by embryonic exposure to either estrogen or

androgens before ED 12 (Ottinger et al, 2001; Schumacher et al, 1989). Again, this

suggests that the development of these mechanisms might also be modulated by a balance

of both gonadal steroids. *In ovo* exposure to androgenic trenbolone could disrupt this

balance and cause immediate morphological differences, and later functional differences,

in the POA. Embryonic exposure to exogenous androgens has already been shown to

disrupt development of the POA. Although the size of the POA is unaltered by

exogenous androgens, the size of the neurons and the volume of their nuclei in the

dorsolateral area of the POA become permanently altered (Castagna, 1999; Panzica et al., 1999).

A balance of the gonadal steroids is needed for appropriate morphological differentiation to occur. Any EDC that mimics or blocks the effects of these steroids may upset this balance and disrupt normal development. The development of some structures may be more resistant or resilient to chemical exposure than others. Morphological, functional, and behavioral measures should all be included in toxicity tests to be able to fully characterize the effects of environmental chemicals on fitness and survival.

Chapter 6: Effects of Androgen-Active EDCs on Vocalization and Motor Behavior

INTRODUCTION

A great deal of research has characterized physiological effects of toxicity in birds exposed to environmental contaminants, however, effects of these chemicals on behavior are less frequently studied (Scott and Sloman, 2004). The use of behavioral measurements as biomarkers of exposure to endocrone disrupting chemicals (EDCs) often gets overlooked in reviews of appropriate and sensitive EDC-exposure endpoints (Fossi et al., 1999). Measures of behavior should be used more often to assess effects of embryonic exposure to EDCs since the developing brain is not only one of the most sensitive targets of EDCs, but also one of the least resilient. Assessment of behaviors that are necessary for survival and fitness is not only a non-lethal method to measure alterations in brain development, but also provides a method of linking physiological function with ecological processes (Scott and Sloman, 2004).

Methods to assess behavior in Japanese quail (*Coturnix japonica*), a model species well suited for toxicity tests (Fair et al., 1999), are limited. Procedures to assess male copulatory behavior are well established and frequently used (Ottinger et al., 2002). Recently, methods to quantify differences in female reproductive behavior have been successfully tested (Domjan et al., 2003). Although a wide variety of tests have been developed to study neurotoxicant-related changes in motor function, a need for simple and specific tests of motor movements exists (Samsam et al., 2004). Wada has measured locomotor activity by floor deflection in adult male Japanese quail (1982). Here, we present a novel modified open field test that is effective in testing an important chick

survival skill: locating and returning to it's brood when separated. Japanese quail are precocial birds, being able to leave the nest and find their own food at day of hatch. It is necessary for chicks to remain with the brood after hatch for protection from predators and for thermoregulation. If a chick becomes separated from its brood, it calls to its conspecifics. Siblings vocally respond to the chick's separation call, which helps the separated individual to locate and return to the rest of the brood.

Androgens have been shown to modulate motor behavior and vocalization in birds. Testosterone and androstenedione induced locomotor activity in castrated adult male Japanese quail (Wada 1984 and 1982). Testosterone, androstenedione, and 5α-dihydrotestosterone have also induced calling in adult male Japanese quail (Wada 1984 and 1982; Adkins and Pniewski, 1978). Other studies have demonstrated testosterone's ability to stimulate vocalization in ring doves and pigeons (Cheng and Lehrman, 1975; Pietras and Wenzel, 1974). These experiments investigated the effects of implanted or injected androgens on adult motor behavior and vocalization. We are not aware of any studies that have examined the effects of embryonic exposure to androgens on these endpoints in chicks.

Most research done with EDCs has focused on the effects of these chemicals on the estrogen and thyroid systems, however, many of these same chemicals also exert strong effects on the androgen system. We have developed a method to assess the influence of EDC exposure on motor behavior and vocalization in week and two-week old chicks that had developed *in ovo* exposed to trenbolone acetate, a synthetic androgen. Trenbolone is used as a growth promoter in beef cattle and has been shown to persist in

the environment long after excretion (Schiffer et al., 2001), where it could enter the food web and act as a potential androgen disrupting chemical.

The main objective of this experiment was to determine if embryonic exposure to trenbolone acetate would disrupt the early Japanese quail survival behavior of being able to locate and return to the brood when separated. The first hypothesis was that *in ovo* exposure to trenbolone would alter the motor behavior of individuals associated with returning to conspecifics after isolation. The second hypothesis was that embryonic exposure to trenbolone would impact individuals' abilities to vocalize and therefore effectively communicate with and locate conspecifics when isolated.

METHODS

Japanese quail, an avian laboratory species well suited for toxicity tests (Fair et al., 1999), used in this study were offspring from a colony at the University of Maryland's Animal and Avian Sciences department, College Park, Maryland, USA. For the first trial, ninety eggs per treatment were randomly selected to receive either sesame oil (control), 0.05, 5, or 50 μg trenbolone acetate (Sigma Chemical Co., St. Louis, MO, USA). For the second trial, eggs were randomly assigned to either control, 5, 50, or 125 μg treatment levels. Environmental levels of trenbolone in bird eggs are unknown. The treatment levels of trenbolone selected for this study were chosen as part of a larger range-finding study. Trenbolone treatments were first dissolved in sesame oil and then injected into yolks at a volume of 20 μl per egg on day four of incubation. Eggs were candled before injections so that only fertilized eggs received treatments. After hatch, quail were randomly assigned by treatment to heated brooders (72x27x25 mm), and diet

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and water were provided *ad libitum*. After differences in vocalization from the first trial were apparent, a second trial was performed to replicate and further quantify those differences. Since the differences were observed at the higher treatment levels, we performed similar yolk injections of trenbolone acetate at 5, 50, and 125 µg per egg.

All measurements were conducted in a modified open field runway test at weeks 1 and 2 posthatch for both trials. Ten quail per treatment were randomly selected for each week's test. Individual quail were separated from their conspecifics and allowed to call and return to the group at the opposite end of a runway (182 cm long) within three minutes. Separated quail were able to see and hear the rest of the chicks that were contained in a 1728cm^3 cage at the far end of the runway. The runway was divided into five lanes that were each 12 cm wide. Motor behavior was assessed by measuring the amount of time it took individuals to reach conspecifics and the number of lanes individuals crossed. If individuals did not reach their conspecifics, the maximum distance individuals traveled within the three minutes was recorded.

Defecation rate is a measurement that has been used to measure stress responses in pigs (Desautes et al., 2002). In this study, the number of defecations made by individuals separated from their conspecifics within the three minutes of testing was also used to assess stress.

In the first trial, vocalization was measured by observing whether or not individuals called to their separated conspecifics or not. Therefore, vocalization for the first trial is expressed as the percentage of individuals per treatment that performed stress vocalizations. Unexpected results in vocalization observed at the higher treatment levels prompted a second trial to be made to confirm the effects of embryonic exposure to

trenbolone on vocalization. Calling behavior in the second trial was quantified at both weeks 1 and 2 of age by measuring the number of calls produced by individuals.

The Statistical Analysis System (SAS Institute, Inc. 1987) was used for all statistical analyses. Assumptions for parametric statistics were examined prior to analysis. Chi-square tests were performed to assess results expressed as percentages. All other measurements were analyzed by two-way analysis of variance, and Tukey tests were used for post hoc pairwise comparisons. Data were analyzed separately by sex when sex-by-treatment interactions were significant.

RESULTS

At week 1 of age, isolated Japanese quail chicks that were embryonically exposed to trenbolone acetate reached their conspecifics faster than controls (Fig. 19; $p<0.05$). Quail that received the 0.05 μg treatment reached other members of their brood about 80% as quickly as controls ($p<0.05$). Chicks that were exposed to 5 and 50 ug trenbolone reached their conspecifics in about one-fourth the amount of time as controls ($p<0.05$ for both). These differences in the amount of time required to reach conspecifics no longer existed when quail were tested at 2 weeks of age. The distance traveled by quail did not significantly differ among treatments for either week; the majority of individuals, regardless of treatment, reached the other members of their brood within the 3 minutes of testing. The number of lanes individuals crossed while traveling towards their conspecifics was also not significantly different among the treatments for either week.
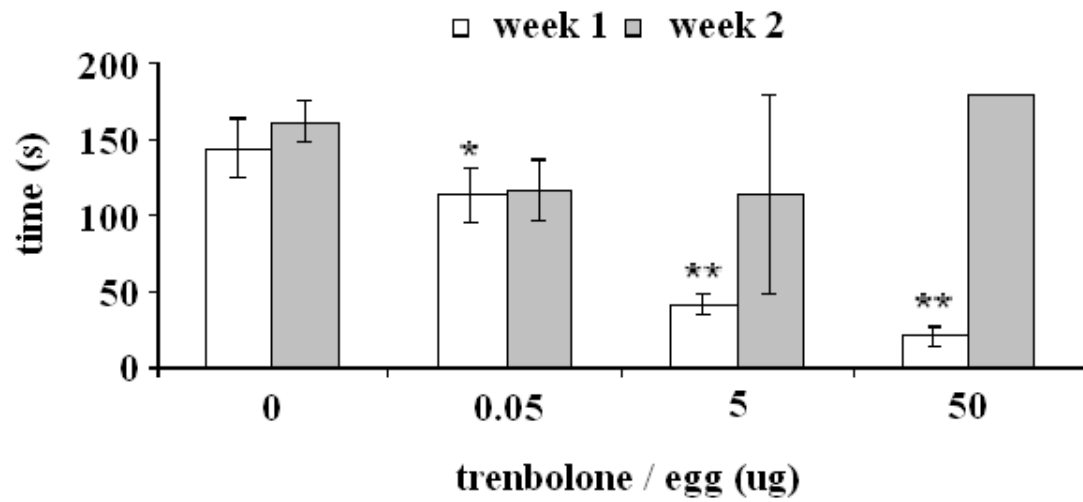
Figure 19. Time required (mean +/- SEM) for isolated Japanese quail chicks to rejoin conspecifics 182 cm away at one and two weeks of age. Significant differences between treatments are indicated by asterisks (p<0.01).

Treatment and defecation were not independent for the week 1 measurements [Fig. 20; $X^2(4, N = 50) = 10.0$). At week 1 of age, no quail from the 50 µg treatment level was observed to defecate within the 3 minute testing period. At week 2, individuals from the 50 µg treatment group defecated a similar number of times as control quail.

In the first trial, treatment and calling were not independent [Fig. 21; $X^2(4, N=50) = 19.6$). Stress vocalizations were reduced in the 5 and 50 µg treatment groups for both weeks one and two. No vocalizations from birds of the 50 µg treatment group were heard by the observer. In the second trial, the number of vocalizations made by individuals were significantly fewer than controls (Fig. 22) when measured at both 1 and 2 weeks of age ($p<0.05$). Zero vocalizations were recorded at the 50 and 125 µg treatment levels.

DISCUSSION

A great deal of previous research has characterized physiological mechanisms of toxicity in animals exposed to contaminants, however, effects of contaminants on avian behavior are less frequently studied. Studies that have examined the effects of EDCs on avian behavior most often exposed subjects to treatments post-hatch. Similarly, studies that have characterized the hormonal mechanisms behind avian behavior have focused on how hormones elicit behaviors in juveniles or adults that have not been embryonically exposed to exogenous hormones. These types of studies that administer hormonal or EDC treatments in feed or implants have shed a great deal of light on how neural pathways are triggered, but they speak little about how the endocrine system influences embryonic development of these circuits.
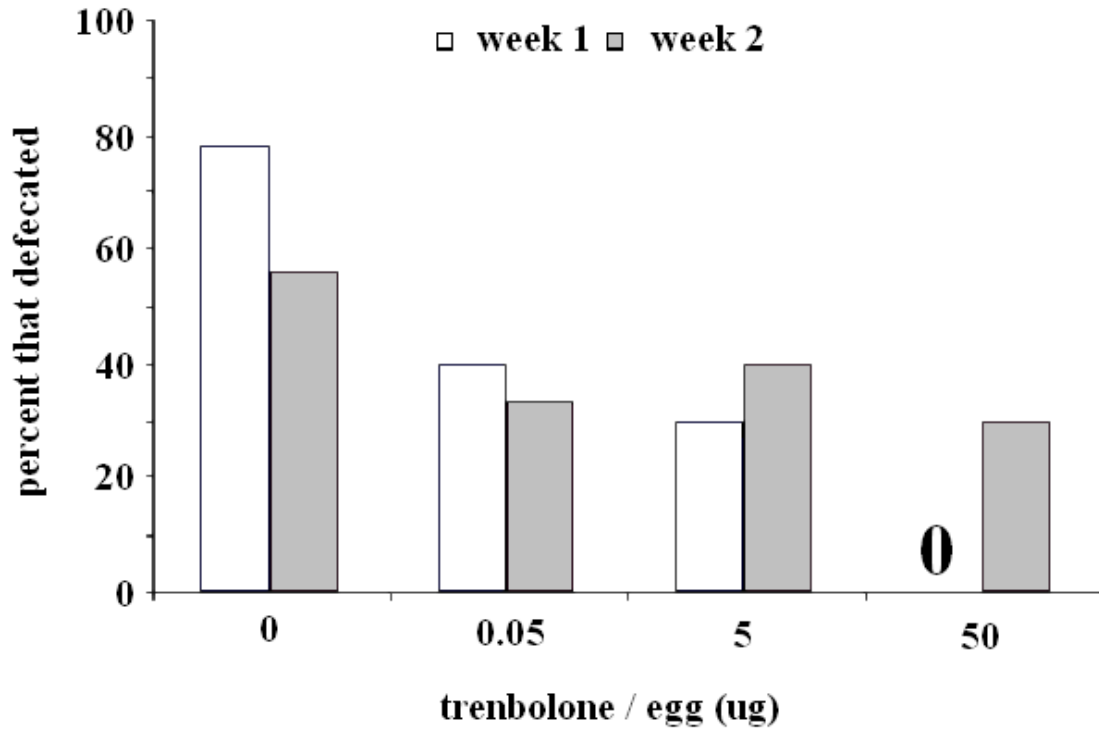
Figure 20. Percent of Japanese quail chicks that defecated during a three-minute motor behavior test at one and two weeks of age.
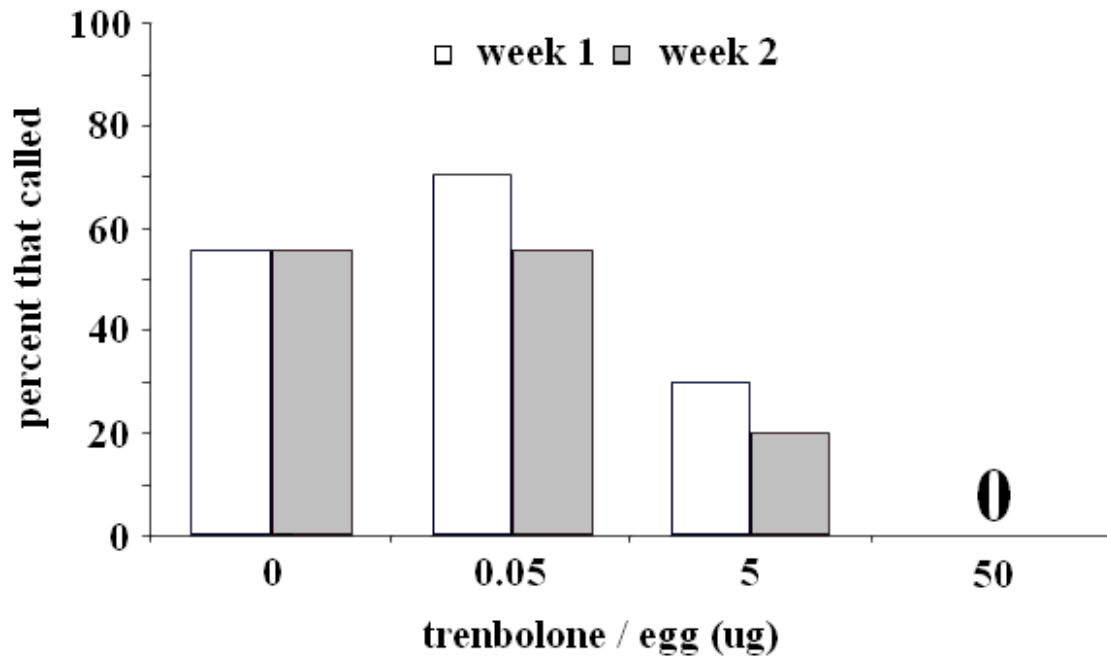
Figure 21. Percent of isolated Japanese quail chicks that called to conspecifics during a three-minute motor behavior test at one and two weeks of age.
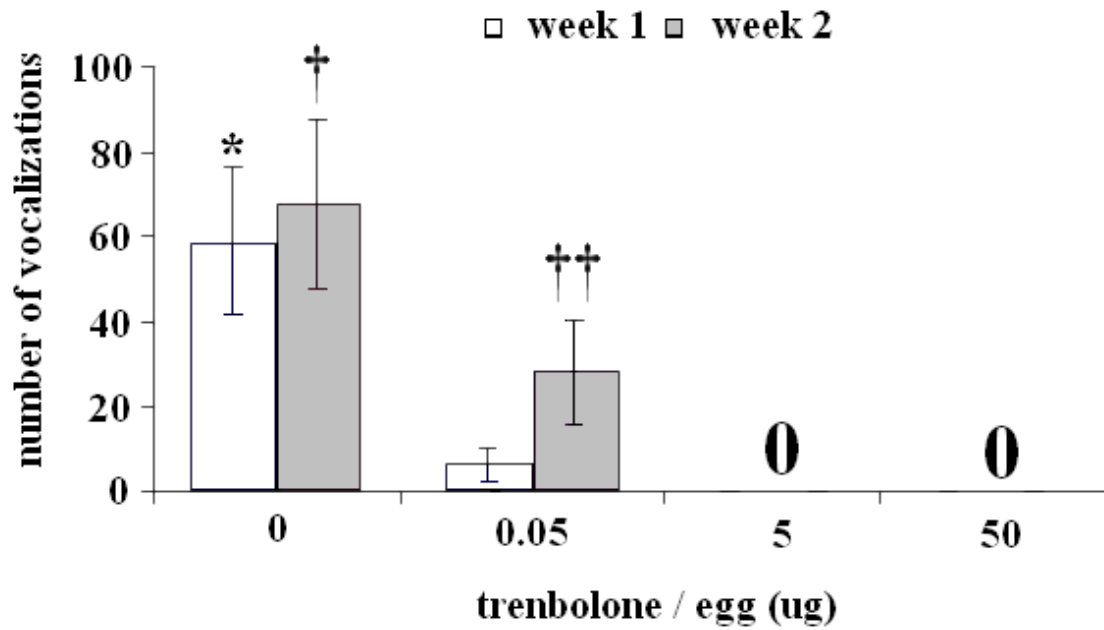
Figure 22. Number of vocalizations (mean +/- SE) made by isolated Japanese quail chicks that called to conspecifics during a three-minute motor behavior test at one and two weeks of age. Significant differences are indicated by asterixes for week one and crosses for week two.

Few studies examining the effects of hormonal influence of sex steroids on avian motor behavior have been published since those of Wada (1982, 1984). In these studies, it was shown that testosterone and androstenedione enhanced locomotor activity in Japanese quail, as measured in light-tight boxes by the number of floor deflections (described in Wada, 1981). The routes of exposure for the androgens were either implants of Silastic capsules or pellets. Treatments were administered to quail that were 7 weeks of age, having been castrated 2 weeks before implantation. Although these studies have shown that locomotor activity is androgen dependent in Japanese quail that were allowed to develop normally as embryos, no inferences can be made here concerning the effects of androgens on the development of the areas of the brain responsible for locomotor activity, possibly around the third ventricle at the medial basal portion (Wada, 1984). If this is indeed an area of the brain responsible for androgen dependent locomotor activity, the results of the current study suggests that it is possible that the development of it is also androgen dependent, or at least sensitive, and therefore susceptible to disruption by embryonic exposure to androgen-active EDCs.

The effect of trenbolone on defecation was not expected. This measurement was made to quantify a stress response. After necropsies of chicks from the higher treatment groups were completed, it was very obvious that reduced defecation was not a behavioral response, but a physiological one. Many of the chicks that did not defecate simply could not. Their colons were impacted with feces, and this was most likely the cause of their death. Thus, the increased rate of defecation at week two at the high levels of trenbolone treatments is most likely due to a survivor effect. Those individuals that survived the

second week were most likely the individuals that were able to defecate. The majority of the individuals with seriously impacted colons probably died within the first week.

The differences in vocalization that were observed in this study could be due to either of two reasons. The intercollicular nucleus of the mesencephalon has been identified as the vocal neural system responsible for producing the distress call in Japanese quail chicks (Yazaki et al., 1997). Neurons from the intercollicular nucleus of adult males have many more dendrites than those of females. This difference suggests that the development of these neurons may be influenced by differences in sex steroids between the sexes, although it has not yet been determined if this difference is indeed caused by testosterone (Yazaki et al., 1999). Testosterone has been shown to affect this vocal neural system during development, modulating the amplitude, frequency, and behavior of the adult male call. Furthermore, following electrical stimulation of the intercollicular nucleus, females were able to produce the male crow four days after subcutaneous implantation of testosterone (Yazaki et al., 1999). So although it is known that testosterone can alter the structure of the intercollicular nucleus in adults and induce male crowing, it is unknown if testosterone is able to modulate this area of the brain for production of the distress call in chicks.

Trenbolone inhibited the production of sound in the quail of the current study, but the authors do not believe that the calling behavior was abolished. Likewise, the authors do not believe that trenbolone caused disruption of development of the intercollicular nucleus in a way that caused inhibition of the functioning of the neural mechanism behind the stress vocalizations. During observations recorded in the second trial, it was noticed that the majority of the birds that were unable to produce sound were straining

and moving their beaks when isolated, resembling the stress response behavior of the control birds. Thus, the behavior remained intact; separated individuals were able to respond to the stress of being separated from their conspecifics, but were unable to effectively vocalize.

Neural projections connect the intercollicular nucleus with the hypoglossal nucleus, the control center of the syrinx (Wild, 1993). The syrinx is the avian vocal cord located at the junction of the trachea into the primary bronchi. Syrinx mass is greater in male zebra finches than females (Lohmann and Gahr, 2000). Although the mechanisms behind this dimorphism are unknown, it suggests a possible role of sex steroids during development. Indeed, most of the research investigating the effects of gonadal steroids on syrinx development has used this species. The zebra finch syrinx has been found to contain androgen receptor mRNA by embryonic day 10, presumably in order to prepare the chick for post-hatch food begging behavior (Godsave et al., 2002). One study has shown that Silastic implants of testosterone in adult zebra finches caused significant increases in syrinx mass and the size of the ventralis and dorsalis syrinx muscles, and implants of flutamide, an anti-androgenic chemical, decreased syrinx weight (Wade and Buhlman, 2000). However, results from the few studies that investigate the effects of gonadal steroids on syrinx development are inconsistent. It is clear that the effects of these hormones on syrinx development is complicated, and it is generally agreed that although they do not appear to be directly responsible for the stimulation and control of the overall process of sexual differentiation of the syrinx, areas connecting the vocalization-modulating areas of the forebrain to the syrinx can be altered by exogenous administration of them (Wade et al., 2002).

Regardless of the mechanisms behind the observed trenbolone-induced suppression of vocalization, one must consider the ecological implications of this effect. The vocalization behavior measured in this experiment is a survival behavior for chicks; suppression of this behavior can obviously increase individual mortality and have detrimental effects on viability at a population level. Suppression of other types of avian vocalizations may also have detrimental, albeit more subtle, population effects. Birds vocalize for a number of critical behaviors to ensure individual survival and fitness: food begging in chicks, mate attraction, sexual selection, and announcement and defense of territories. Although the measurement of vocalizations in this experiment was not initially the main drive of the study, the importance of this serendipitous finding is clear when considering the potentially devastating impacts of androgen active EDCs on this function at population levels. Very little is known about environmental effects of trenbolone; although considered to be safe, most of the data collected by industrial laboratories concerning the effects of this chemical on embryonic development are unpublished (IPCS and CCOHS, www.inchem.org/documents/jecfa/jecmono/v25je08.htm). From the results of this study, the authors strongly encourage that its status as "non-teratogenic" should be reconsidered, and effects of this chemical and other androgen-active EDCs on avian vocalization assessed.

Chapter 7: Effects of Androgen-Active EDCs on Growth and Developmental Stability

INTRODUCTION

Growth and developmental stability are often used as measures of chemical stresses experienced during development. Individual growth rates have been measured in a number of taxa to assess the effects of chemical pesticides, biological pesticides, and heavy metals on development (Hatakeyama et al., 1997; Willingham, 2001; Spahn and Sherry, 1999; Norton et al., 2001). Disruption of early development can have permanent effects on the adult individual that may affect the population to which the individual belongs. In birds, even reduced growth experienced during the first two weeks of development may have important consequences at the population level (Norton et al., 2001; Fairbrother et al., 1994).

Fluctuating asymmetry (FA) in morphology has often been used to indicate stress that was experienced during an individual's development. Since the development of morphological structures on each side of a bilaterally symmetrical animal is under genetic control, it is assumed that they would develop identically as they are products of the same genome (Leary and Allendorf, 1989). Although the relevance of patterns of differences in symmetry in bilateral organisms is often debated (Palmer and Strobeck, 1986), it is generally recognized that FA, or deviations in aspects of the right and left sides of an organism, is almost entirely environmentally induced (Bortolotti and Gabrielson, 1995).

FA is only one type of morphological asymmetry; antisymmetry and directional asymmetry are two other types first described by Van Valen (1962). Antisymmetry is characterized by a bimodal distribution of right/left differences about a mean of zero, and

directional asymmetry is characterized by a normal distribution of right/left differences, except that the mean is displaced positively or negatively from zero (Palmer and Strobeck, 1986). FA is characterized as having a normal distribution of right/left differences with a mean of zero. Antisymmetry and directional asymmetry have a genetic component, but FA is thought to be caused purely by environmental factors (Novak et al., 1993), and as such is most often used to indicate the effects of environmental stress on development.

Only a limited number of studies have tested the use of FA as a potential indicator of embryonic exposure to environmental contaminants, and most studies have only examined correlational relationships between pollutants and FA. Eeva et al. (2000) reported increased asymmetry of the tarsus length in pied flycatchers (*Ficedula hypoleuca*) and the primary feather length in great tits *(Parus major)* in individuals found closer to a copper smelter. This suggested a positive association between heavy metal exposure and FA. FA was higher in common shrews (*Sorex araneus*) from areas of high metal contamination compared to shrews collected at reference sites (Pankakoski et al. 1992). One study directly linked increasing FA with increasing chemical exposure (Mpho et al. 2001). In this case, FA in wing characters of male mosquitos (*Culex quinquefasciatus*) increased as levels of organophosphate treatments increased. This is the only study that we are aware of, however, to link causality to pesticide exposure and FA in spite of the use of over 100,000 chemicals as pesticides worldwide (Allenbach et al., 1999).

The objective of this study was to examine the effects of a one time *in ovo* exposure of androgen disrupting chemicals (ADCs) on growth and developmental

stability as measured by FA in the Japanese quail (*Coturnix japonica*). ADCs target androgenic systems and androgen dependent responses, resulting in altered synthesis, release, or action of androgens. Embryos were exposed to either an androgenic ADC, trenbolone acetate, or an anti-androgenic ADC, p,p'-DDE [ethylene, 1,1-dichloro-2,2-bis(p-chlorophenyl)]. Trenbolone acetate is a synthetic androgen used as a growth promoter in beef cattle. DDE is the major metabolite of the pesticide p,p'-DDT [1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane] that acts as an androgen receptor blocker that is chemically more stable and biologically more persistent in than its parent compound (You, 2000). This study examines embryonic ADC exposure and FA in an avian model to validate FA as a potential biomarker for endocrine disrupting chemical (EDC) exposure.

METHODS

Japanese quail, an avian laboratory species well suited for toxicity tests (Fair et al., 1999), used in this study were offspring from a colony at the University of Maryland's Animal and Avian Sciences department, College Park, Maryland, USA. All FA measurements were performed on ten male and ten female day old chicks per treatment. Quail that were measured for growth were randomly assigned by treatment to heated brooders (72x27x25 mm). Diet and water were provided *ad libitum*. Chicks received Purina Game Bird Startena BMD 50 diet, and adults received Purina Game Bird Breeder Layena Complete Ration (St. Louis, MO, USA).

Ninety eggs per treatment were randomly selected to receive either sesame oil (control), 20.0 or 40.0 μg of p,p'-DDE, or 5 or 50 μg trenbolone acetate (Sigma Chemical

Co., St. Louis, MO, USA). Chemical treatments were dissolved in sesame oil and injected into yolks at a volume of 20 μl on day one of incubation. The low dose of DDE reflects environmentally relevant levels that should not affect reproduction, but should have an effect on development; the high dose reflects environmentally relevant levels that have been found to affect reproduction (Nisbet 1989, Henny and Herron 1989, Custer and Mitchell 1987, Henny et al. 1984, Custer et al. 1983, and White et al. 1988). Levels of trenbolone in yolks in the environment are unknown; the levels of trenbolone used in this study were determined as part of a larger range finding study.

We measured body weight every other day to the nearest 0.01 g by digital balance (Mettler Type MT5, Mettler, Hightstown, New Jersey, USA) and right tarsus length (section of the foot between the metatarsus and leg) and culmen length (from the tip of the upper mandible to the beginning of the cere) every third day to the nearest 0.01 mm by digital calipers (Traceable, Friendswood, Texas, USA) from day one until day 29. Ten individuals per treatment were randomly selected for measurements.

The characters used in the FA measurements included the tarsus, the radius, the zygomatic process, and the premaxilla. These measurements were made on the bodies of day 1 birds to the nearest 0.01 mm with the same digital calipers as described above. Each bilateral trait was measured twice by the same individual with an interval of 5-8 days between the two measurements as suggested by Merila and Bjorklund (1995) to help avoid measurement error.

The Statistical Analysis System (SAS Institute, Inc. 1987) was used for all statistical analyses. Assumptions for parametric statistics were examined prior to analysis. The growth measurements were analyzed by two-way analysis of variance

(ANOVA). Two-way ANOVAs with two repeated measurements were made by the same person for all the FA measurements except wing web thickness.

RESULTS

Individuals that were embryonically exposed to both treatment levels of DDE experienced steady increases in body weight and tarsus and culmen lengths. No differences were observed in body weight at day of hatch. Similarly, no differences were found among treatments for growth as measured by body weight and tarsus and culmen lengths over time. Also, no significant differences were observed among DDE treatments in FA of the tarsus, radius, zygomatic process, or premaxilla.

Similarly, quail that were embryonically exposed to trenbolone had no significant differences in body weight at day of hatch. From day 5 through day 19, however, quail that were exposed to 50 μg trenbolone exhibited lower body weights than controls (p<0.01; Fig. 23). On day 19, quail that were exposed to 5 μg trenbolone also weighed significantly less than controls (p<0.05). No significant differences were observed in tarsus or culmen lengths at day of hatch for quail exposed to trenbolone (Fig. 24 and 25), however tarsus lengths from chicks exposed to 50 μg trenbolone grew at a shorter rate from day 8 until 19 (p<0.007; Fig. 2). Growth of the culmen in birds treated with 50 μg trenbolone was reduced by day 19 (p=0.01; Fig 3). No differences among treatments were observed with FA in trenbolone treated birds.

Effects of Tb on Body Weight

Figure 23. The effects of a one time embryonic exposure of trenbolone acetate at day one of incubation on growth from day of hatch until day 19 as measured by body weight (mean +/- standard error). Treatment levels include control (sesame oil only), low (5.0 μg), or high (50.0 μg).

tarsus growth

Figure 24. The effects of a one time embryonic exposure of trenbolone acetate at day one of incubation on growth from day of hatch until day 19 as measured by length of the right tarsus (mean +/- standard error). Treatment levels include control (sesame oil only), low (5.0 μg), or high (50.0 μg).
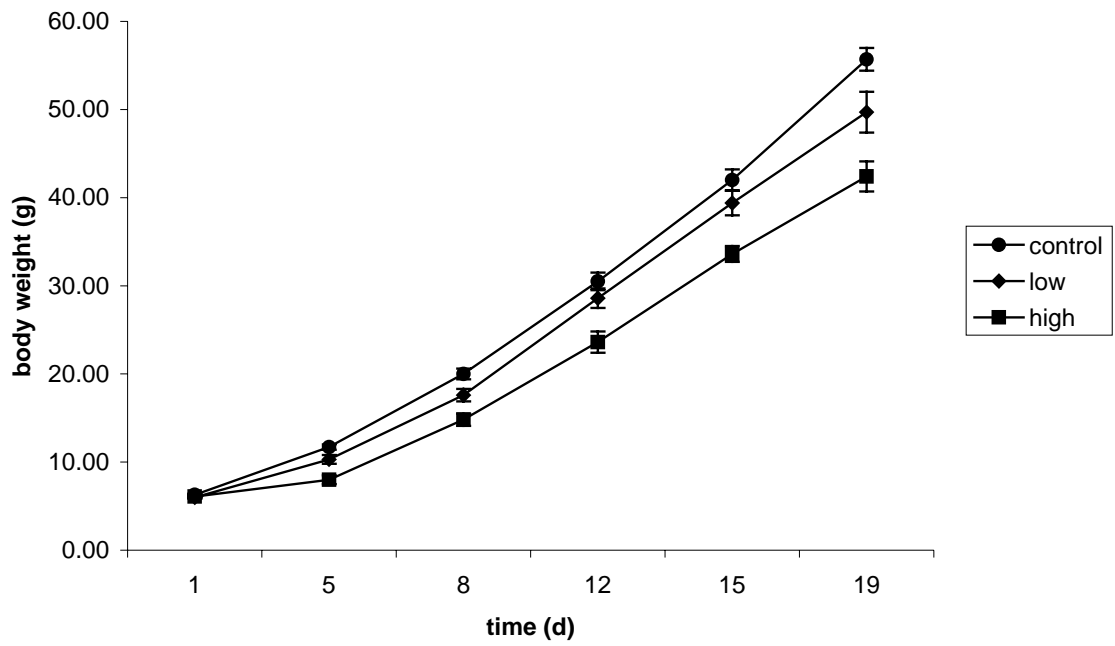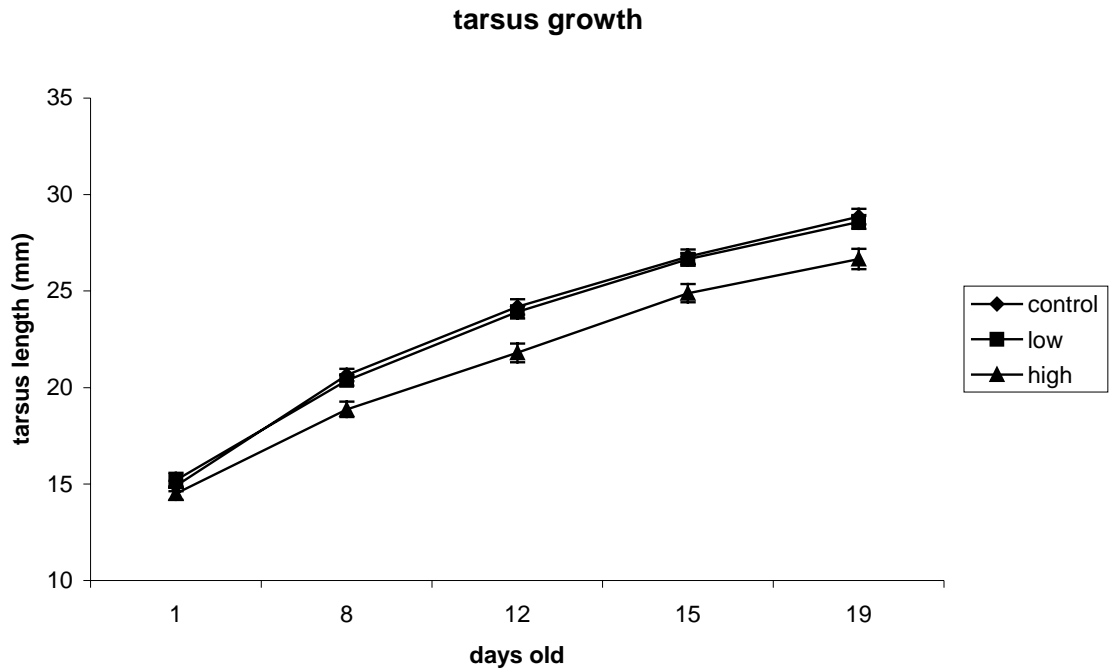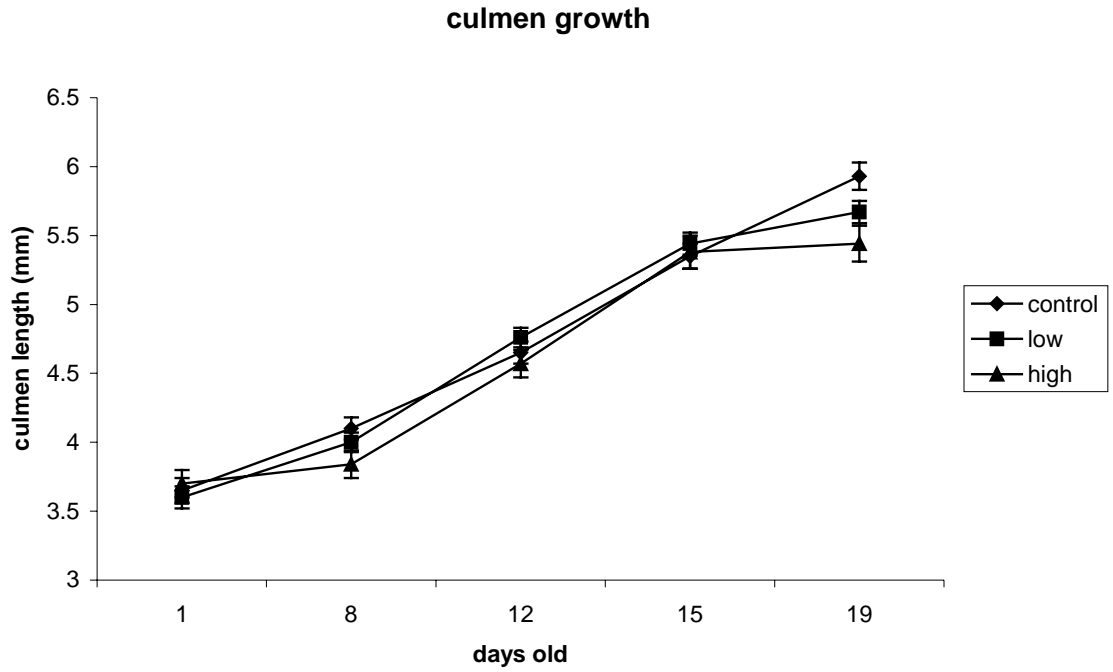
culmen growth

Figure 25. The effects of a one time embryonic exposure of trenbolone acetate at day one of incubation on growth from day of hatch until day 19 as measured by culmen length (mean +/- standard error). Treatment levels include control (sesame oil only), low (5.0 μg), or high (50.0 μg).

DISCUSSION

The lack of significant differences observed with the DDE treatments suggests that our treatment levels were not high enough to affect developmental stability. However, other measurements taken from the same birds showed that behavior and other androgen dependent characteristics had indeed been affected by treatments (Quinn et al., unpublished data). Specifically, weights of the bursa of Fabricius, a primary lymphoid organ, were significantly larger in day old DDE-treated birds than in controls. Total leukocyte numbers were also significantly higher in the day old DDE-treated birds than controls. Female quail in the high DDE dose group reached puberty approximately one week before the other treatments. Male sexual behavior was also disrupted by the one time in ovo exposure to DDE. Treated males attempted to mount females half as many times than controls and required approximately twice the amount of time to accomplish successful cloacal contacts with females.

Body mass is often the only measurement of growth taken in the few studies that have examined the effects of DDE on growth. Most experiments have not examined effects of DDE exposure on growth as measured by length of skeletal characters. If growth of skeletal characters is to be affected by DDE, growth of these characters should be sensitive to androgens. Therefore, the second question to arise was, what kind of role do androgens have on skeletal growth? Although the mechanism by which androgens induce skeletal growth is not yet fully understood, it is known that they interact with other gonadal steroids, growth hormone (GH), and insulin-like growth factor-I (IGF-I) on the epiphyseal growth plate (Mauras et al., 1996; Maor et al., 1999; Zmuda et al., 2001).

Androgens stimulate GH secretion by their aromatization to estrogen (Keenam et al., 1993). Testosterone directly stimulates local production of IGF-I and IGF-I receptors in chondrocyte cell layers of the murine mandibular condyle, which may or may not necessarily represent growth centers of longitudinal bones (Maor et al., 1999). Further studies are needed to understand the individual actions and interactions of androgens and other hormones, growth factors, and receptors.

Although androgens are known to induce skeletal growth, exposure to the synthetic androgen trenbolone acetate inhibited all measures of growth. Observations and necropsies of chicks indicated that trenbolone may have caused disruption in the function of the gastrointestinal tract (GI). Chicks were often observed to have a large build-up of fecal material around the cloaca. In some cases, it appeared to make defecation difficult. Necropsies of chicks that died in between day 1 and 19 revealed that birds that had a large build-up of feces around the cloaca also had an abnormally large amount of fecal material concentrated in the end of the colon. The inhibition of growth, therefore, could have been caused more by trenbolone's effects on the development and functioning of the GI tract, rather than its direct effects on skeletal growth.

Again, measures of FA in trenbolone also revealed no significant differences among treatments. An important consideration is that the degree of measurement error in skeletal measurements is reported to be often fairly high (Lougheed et al., 1991). As such, it is not surprising for estimates of FA involving morphometric characters to be very sensitive to measurement error (Merila and Bjorklund, 1995). Despite many papers stressing the importance of considering measurement error in studies of FA, studies often either continue to neglect accounting for it or do so incorrectly (Merila and Bjorklund,

1995). We accounted for the bias due to measurement error by using the two-way ANOVA with two repeated measurements, as suggested by Merila and Bjorklund (1995) and Palmer and Strobeck (1986).

It is suggested that FA has good potential as a biomarker in ecotoxicologic research when used correctly (Palmer, 1996). Many FA measurements can be obtained through noninvasive techniques. This is beneficial when working with threatened or endangered species, or when employing a mark/recapture program. Some of the most often used avian measures of FA include tarsus length, feather length, and wing length. (Lens et al., 2002; Ohlsson and Smith, 2001; Anciaes and Marini, 2000, Eeva et al., 2000). Fair et al. (1999) used the masses of primary feathers as FA measurements in Japanese quail. Since these measurements require no specialized equipment, they are easy to perform and are cost effective. FA also appears to be generally applicable to a wide range of circumstances (Allenbach et al., 1999); it is a measurement that can be used on a wide variety of species and is affected by a variety of stressors.

In conclusion, although growth and FA can serve as reliable biomarkers in a wide variety of circumstances, these measures do not appear to be sensitive indices of endocrine disruption by DDE or trenbolone acetate at the exposure levels used in this study in Japanese quail.

Chapter 8: Conclusion

This study had evolved greatly from its original direction of focusing solely on the effects of androgen-active EDCs on the development and function of the immune system. We were very opportunistic in taking advantage of the animals and treatments used by including measures of reproduction, growth, developmental stability, motor behavior, and vocalization. Some findings were surprising and have fostered new directions in our research, including consideration of the effects of EDCs on chick vocalization. Thus, the main challenge in completing this project has become compiling all of the information in a clear manner that will be most useful to the toxicology and neurotoxicology communities. One of the most pressing current needs is the determination of reliable biomarkers of exposure to environmental contaminants in avian species. Because the traditional toxicological measures appear to be largely insensitive to the more subtle effects of EDCs on endocrine systems, it has been critical to evaluate appropriate endocrine-based measures that would serve as indices in laboratory studies and eventually for wild populations. Certainly in our own studies, we have determined specific various measures that have proven to be more sensitive to EDC exposure than other basic toxicological measures. Also, some EDC associated alterations in development that were observed in chicks had disappeared in adults. Thus, adult birds often appear to adapt to low levels of contaminant exposure, and as such, traditional reproductive measures of toxicity conducted in adults may not be adequate to assess overall effects on fitness.

In these experiments, immune measures proved to be responsive end points to androgen-active compounds. The most sensitive morphological measures of exposure in

this study were those of the bursa of Fabricius. Initial alterations in development observed in bursas from chicks treated with either p,p'-DDE or trenbolone acetate were immediately obvious. Few articles have been published that describe observed differences in bursal morphology following exposure to exogenous androgens. Those that exist were very descriptive and only provided qualitative data. Once the bursas were known to appear different, the next challenge soon became to answer the question, *how different are they?* Quantitative measurements would be the only way to accurately answer this question, and none have yet been reported in the current literature.

In determining the best way to make quantitative measures of bursal morphology, I selected the parts of the bursal that played the strongest role in B cell maturation. Since B lymphocytes mature in the follicles, measurements of these structures were the obvious first choice, and indeed, they were very sensitive endpoints. Although not quantified, another sensitive bursal biomarker of exposure was the lobe-shape of the bursal folds or plicae, which was distorted or absent in most of the day-old bursas from both DDE and trenbolone-treated quail. It should also be noted that although bursal morphology appeared to be greatly altered in chicks, differences greatly diminished in adult bursas. This suggests that, the bursa may be fairly resilient to embryonic EDC-induced disruption, provided that not further exposure occurs post-hatch. Therefore, although few differences existed in adult bursal morphology, this biomarker of exposure may be most appropriate in chicks.

Reproductive behavior also proved to be a sensitive and appropriate biomarker for androgen-active EDC exposure. Both DDE and trenbolone acetate caused significant reductions in the number of attempts to mount females. DDE exposure resulted in an

increase in the amount of time to achieve successful copulations, and trenbolone caused a reduction in the number of successful copulations achieved. All of these differences were observed in the first of three tests only. This suggests that males were able to compensate for the initial behavioral impairment through experience. However, it should be noted that the males and females were confined in a small cage throughout the tests. Males that were finally able to achieve a successful mating often did so with great difficulty, having to chase the female around the cage for most of the three minutes required of the test. Males might not have this type of opportunity in the wild to gain the experience needed to overcome their initial behavioral impotence. Likewise, if females in the wild deem a male unacceptable for mating, they would be free to leave the male to seek out a more fit suitor. By its very nature, non-random mating strategies are often very competitive. Therefore the resilience that we have observed in male copulatory behavior may not be fully realized in a natural environmental setting.

Onset of puberty may also be a sensitive biomarker for androgen active EDC exposure. Timing to sexual maturation becomes very important to an individual's potential to pass its genes on to future generations. Commonly, individuals who mature faster gain a reproductive advantage over those who mature later. This natural variation in timing to onset of puberty plays a role in contributing to a population's fitness. If exposure to EDCs causes disrupts the natural variability in a population's timing to onset of puberty, alterations in the population's fitness may result if less fit individuals are able to mature faster than more fit ones. Some studies show correlations between onset of reproductive ability and egg quality (Gous et al., 2000; Joseph et al., 2002). Most of these studies, however, were conducted for application in the poultry industry, and as

such, results from these studies can only be used to imply ecological implications to differences in onset of sexual maturation. Most of these types of studies done for the poultry industry measure egg weight and number of eggs laid and interpret the results in regard to effects on egg efficiency. Eggs from these studies are seldom incubated. Incubation of the eggs would allow the assessment of fertility and viability, and would be, therefore, more appropriate in determining the effects of onset of puberty on a population's fitness.

The most surprising finding in this study was that embryonic exposure to trenbolone acetate inhibited chick vocalization. It was originally hypothesized that motor behavior would have been more affected than vocalizations. Also, it was initially thought that any differences in vocalization would be due to behavioral reasons and not mechanical ones, meaning that we did not expect the ability of calling to be completely abolished. It also appeared that calling was more strained in the lower treatment levels, and that the volume of the calls produced by these quail was lower than those from control birds. However, we were unable to quantify these apparent differences. Although there was little room in this dissertation to allow for the exploration of possible mechanisms behind the inhibition of vocalization, the results from this part of the study do show that this measure was a sensitive biomarker for exposure to trenbolone. Thus potentially, vocalization may be a useful measure of exposure to other classes of EDCs.

This last statement begs the question, what makes a biomarker "useful"? Biomarkers are useful when they can accurately indicate exposure to environmental chemicals. As already discussed, endpoint sensitivity is often used to assess a biomarker's usefulness. Sensitive endpoints are desirable because they are more likely to

be able to demonstrate lower levels of contamination. Another factor that determines the usefulness of biomarkers is the ecological implications that can be drawn from them. Traditionally, laboratory experiments have primarily used biomarkers that are able to reveal or attempt to predict something about the effects of a chemical on the fitness of a population, such as reproductive measurements. Fitness is the relative contribution of individuals' alleles to the gene pool of future generations. Therefore, fitness contributes to a population's survivorship, which is benefited by having the heartiest individuals successfully reproduce. In the current study, the most sensitive indicators of exposure to androgen active EDCs that can be used to assess potential effects on a population's fitness are those that measure reproductive behavior. Physiological reproductive measures in this study revealed no significant effects; gonadal development appeared to not be impacted by embryonic exposure to either DDE or trenbolone acetate. Intact and functional gonads do not make any contributions to fitness, however, if the performance of reproductive behavior is impaired. Also, with the potential of some of the more resilient biomarkers used in this study only being appropriate in chicks, behavior appears to be an even more reliable fitness-type biomarker since tests of behavior were conducted in adults. As such, future biomarkers that assess effects of chemicals on reproduction may include measures of the neuroendocrine systems responsible for the activation of these behaviors.

Besides testing fitness, a measure of a population's potential survivability, studies should also focus more on subtle measures of individual survivability. Environmentally relevant exposure levels to EDCs can oftentimes be quite low, and the resulting effects might not be strong enough to cause effects that would impact measures of fitness such as

reproduction. Biomarkers that assess the potential for individual survivability are a necessary complement to the traditional fitness measures because reproductive ability means little if individuals do not live long enough to reach puberty. As such, measures of individual survivability, such as immunocompetence, are challenging in a laboratory setting. In the environment, animals are often exposed to larger numbers and varieties of pathogens than in a laboratory setting, which is oftentimes relatively cleaner. As such, individuals that are immunocompromised and might not have made it to adulthood in the wild might live longer in a laboratory study, and not provide the most accurate measure of potential survivability. In this study, the bursal morphology measures, as discussed above, were the most appropriate biomarkers that addressed potential individual survivability.

It is essential that studies that attempt to assess the potential effects of EDCs in birds be able to measure the impact of these chemicals on both the embryonic organization and adult activation of endocrine-mediated systems. Currently, guidelines for avian toxicity tests are still debated heavily by the Organisation for Economic Co-operation and Development (OECD), and no established methods for such tests have been accepted yet. The current study has identified sensitive and reliable biomarkers for exposure to androgen-active EDCs. Since one of the current areas of debate within the OECD is the development of an appropriate avian two-generation toxicity test, the next step is to determine if the biomarkers identified in this study are as reliable in a subsequent generation. This is an exciting time in toxicology, where harmful effects of EDCs are being identified faster than the mechanisms behind them. I believe that the identification of reliable endpoints can be realized in the near, if not immediate, future.

The majority of energy and efforts should be used in ascertaining how to amend and prevent the harmful effects of EDCs. The biomarkers that are identified now will help to direct where these efforts are focused.

Appendices

I.   Effects of DDE on hatchability

	DDE µg/egg		
	control	20	40
# fertile eggs injected	95	95	95
enbryonic + pipping mortality	41	35	35
hatchability	57%	63%	63%

II.   Effects of trenbolone acetate on hatchability

	trenbolone µg/egg				
	control	0.05	0.5	5	50
# fertile eggs injected	84	80	84	80	120
enbryonic + pipping mortality	19	16	14	17	65
hatchability	77%	80%	83%	79%	46%

III.   Immune Results Summary

	DDE		trenbolone	
	chick	adult	chick	adult
bursa weight	?	NE	?	NE
spleen weight	NE	NE	NE	NE
follicle number	?	NE	?	?
follicle size	NE	NE	?,?	NE
bursal cell area	NE	NE	NE	NE
spleen morphology	NE	NE	X	X
total leukocytes	?	NE	NE	X
differential counts	NE	NE	H:L ?	X
serum IgG	NE	X	?,?	X
humoral response	X	NE	X	NE
cell-mediated response	X	NE	X	NE

NE = no effect
X = not measured

IV.   Reproduction Results Summary

	DDE	trenbolone
male onset to puberty	NE	?
female onset to puberty	?	NE
ovarian follicle counts	NE	NE
foam gland size	NE	?
ovarian weight	NE	NE
testes weight	NE	NE
sperm motility	NE	NE
sperm penetration	NE	NE
latency to mount	NE	NE
latency to successful sex	?	NE
number of mount attempts	?	?
number of cloacal contacts	NE	?

NE = no effect
X = not measured

V.   Growth and Fluctuating Asymmetry Summary

	DDE	trenbolone
body weight	NE	↓
tarsus length	NE	↓
culmen length	NE	↓
FA tarsus	NE	NE
FA radius	NE	NE
FA zygomatic process	NE	NE
FA premaxilla	NE	NE

VI.   Motor Behavior and Vocalization Summary

	trenbolone
time to reach conspecifics	↓
distance traveled	NE
lines crossed	NE
vocalization - trial 1	↓
vocalization - trial 2	↓
defectaion	↓

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