EFFECTS OF THE SOY PHYTOESTROGEN GENISTEIN

ON THE REPRODUCTIVE DEVELOPMENT

OF IMMATURE FEMALE

BROILER CHICKENS

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THE EFFECTS OF THE SOY PHYTOESTROGEN GENISTEIN ON THE REPRODUCTIVE DEVELOPENT OF IMMATURE FEMALE BROILER CHICKENS

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THESIS ABSTRACT

THE EFFECTS OF THE SOY PHYTOESTROGEN GENISTEIN

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Lindsay Marie Stevenson

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Developmentally inappropriate exposures to estrogenic compounds are known to alter morphology and function of the reproductive tract in various species. Chickens are continually exposed to the relatively potent estrogenic soy isoflavones through the diet. It has been shown that the primary soy isoflavone genistein induces proliferation of the chick oviduct. However, information is lacking as to the specific reproductive tract developmental effects of genistein exposure in chicks.

Three experiments were done to compare specific oviduct morphological and functional responses to genistein exposure with responses elicited by a classical estrogen, diethylstilbestrol (DES) in female broiler chicks. To avoid the effects of dietary soy

isoflavones, the experimental diets were formulated with dried egg white, rather than the usual soybean meal, as a protein source. These experiments examined the effects of genistein in the on the morphology and growth of the oviduct and functional responses to estrogen consisting of plasma vitellogenin content and oviductal ovalbumin synthesis.

From the three experiments, it was determined that genistein acts as a weak estrogen in the immature female chick. It has effects on the growth and morphology of the oviduct. Genistein can also induce the synthesis of estrogen-dependent secretions in the chick including vitellogenin and ovalbumin.

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This manuscript is dedicated to the author's parents, John and Colleen Stevenson, and siblings, Nichole and Matthew, for always being there.

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I. INTRODUCTION

Isoflavones, from soy and other plant sources are of interest because of their estrogenic, antifungal, and antibacterial activities. Soybeans and soy-based products are the most significant sources of isoflavones for humans and livestock (Kurzer et al., 1997). Soybeans contain three major isoflavones: genistein, daidzein, and glycitein. Processing of soybeans for livestock feed does little to alter isoflavone content of the resulting meal. Defatted soybean meals will contain essentially all of the isoflavones present in the starting soybeans (Eldridge and Kwolek, 1983). However, alcohol extraction can be used to extract isoflavones and produce low isoflavone soy protein products.

Genistein is the primary soy isoflavone and shares structural features with the animal estrogen, 17-β estradiol. This structural similarity allows genistein to bind to estrogen receptors and sex hormone binding proteins. Through this binding to estrogen receptors, genistein can exert both estrogenic and anti-estrogenic activity (Dixon and Ferreira, 2002). Genistein can also displace bound estrogen and testosterone from human sex steroid binding protein, affecting clearance rates of androgens and estrogens, and therefore the availability of the hormones to target cells (Dixon and Ferreira, 2002).

The estrogenic activity of genistein and other isoflavones, with the possibility of both positive and negative effects on human health, have made them subjects of considerable research interest. Diets containing high levels of isoflavones are known to

negatively affect the fertility of livestock and laboratory animals. As with livestock feeds, most laboratory animal feeds contain soybeans or alfalfa, which are rich in isoflavones (Boettger-Tong et al., 1998; Degen et al., 2002).

The first published research on the reproductive effects of isoflavones showed that ewes suffered from permanent infertility after grazing on high phytoestrogen clover (Bennetts et al., 1946). Since then, isoflavones including genistein have been found to have both inhibitory and stimulatory effects on reproductive function in various laboratory studies. This suggests that it has a biphasic, dose-related action such as causing progesterone release and induction of signal transduction pathways in some organs or tissues but not others (Makarevich et al., 1997). Considering the ubiquitous exposure of livestock and poultry to the estrogenic activity of soy isoflavones, it is important to understand how these compounds affect the reproductive development of these animals.

II. LITERATURE REVIEW

ESTROGENS

Endogenous Estrogens

Endogenous estrogens are members of the steroid hormone super-family. This hormone family includes estrogens, androgens, and progestagens (Saunders, 1998). Steroid hormone formation relies mostly on exogenous cholesterol. Cholesterol has a 27-carbon skeleton that is derived from acetyl-coenzyme A. The initial stage in steroid biosynthesis is the conversion of cholesterol to the C21 compound pregnenolone, and the loss of the 6-carbon fragment (Goldstein and Sites, 2002).

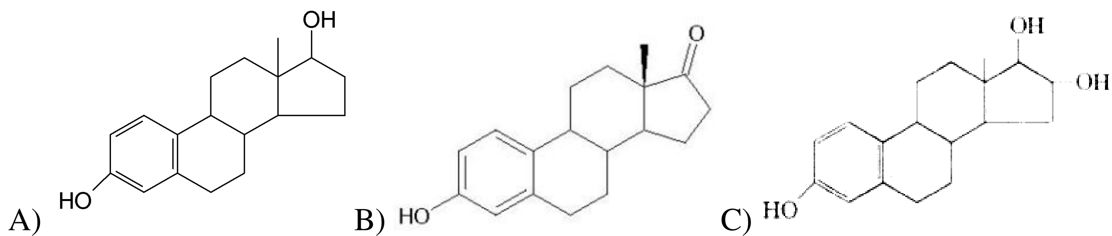


Figure 1: Structures of endogenous estrogens: A) Estradiol, B) Estrone, and C) Estriol.

Estradiol (17β-estradiol) (Figure 1, A) is the predominant estrogen during the reproductively active period of most female vertebrates and is mainly secreted by the ovaries (Bennink and Boerrigater, 2003). It has the greatest bioavailability of the endogenous estrogens (Korach et al., 1997). In humans, esterone (Figure 1, B) becomes

the main estrogen after menopause. It is synthesized in adipose tissue from adrenal dehydroepiandrosterone (Bennink and Boerrigater, 2003). Estradiol can also be reversibley oxidized into esterone (Birkhauser, 1996). The bioavailability of estrogen is reduced 10-fold when it is metabolized into esterone (Korach et al., 1997).

In humans, both estradiol and estrone can be converted into estriol (Figure 1, C), the other endogenous estrogen (Birkhauser, 1996). It is produced in large quantities by the placenta during pregnancy (Bennink and Boerrigter, 2003). Estriol is biosynthesized through a process that is independent of estradiol (Lieberman, 1996). During pregnancy, both estradiol and estriol contribute to uterine growth, placental development, parturition, and the development of the mammary gland (Bennink and Boerrigter, 2003).

Exogenous Estrogens

Estrogenic compounds can come from a number of different sources. Naturally occurring estrogens include compounds that are found in plants and the steroidal estrogens found in humans and animals. Anthropogenic sources of estrogens include synthetic estrogens used in medicine that are excreted into the environment and chemicals that were synthesized for another purpose and then found to have estrogenic activity (Burton and Wells, 2002). The major classes of natural and synthetic estrogens are listed in Table 1.

Environmental estrogens or “xenoestrogens”, have been implicated in the pathogenesis of hormonally treated cancers, male infertility, and abnormalities of both the male and female reproductive tracts (Burton and Wells, 2002). They can interact with estrogen receptors and induce a response that mimics stimulation by endogenous estrogen. The xenoestrogen can also bind to the receptor and produce an inactive

receptor:ligand complex that inhibits the function of the endogenous estrogen (Korach et al., 1997).

Table 1: Natural and Synthetic Xenoestrogens (Korach et al., 1997)

Natural	Synthetic
Flavonoids (phytoestrogens)	Organochlorines
Flavones	Polychorinated biphenyls (PCB)
4’, 7-Dihydroxyflavanone	2’, 5’-Dichloro-4-hydroxybiphenyl
Naringenin	2’, 4’, 6’-Trichloro-4-hydroxybiphenyl
Flavones	2’, 3’, 4’, 5’-Tetrachloro-4-hydroxybiphenyl
Apigenin	Polychlorinated dibenzo-p-dioxins (PCDD)
4’, 5-Dihydroxyflavone	2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin
4’, 6-Dihydroxyflavone	Polychlorinated dibenzofurans (PCDF)
Flavnols	2, 3, 7, 8-Tetrachlorodibenzofuran
Kaempferol	2, 3, 4, 7, 8-Pentchlorodibenzofuran
Hydroxychalcones	Dichlorodiphenylethanes
Phloretin	o, p’-DDT
Isoliquiritigenin	o, p’-DDE
4, 4’-Dihydroxychalcone	o, p’-DDD
Isoflavonoids	Methoxychlor
Isoflavones	Hexachlorocyclohexanes
Genistein	Lindane
Daidzein	Cyclodienes
Formononetin	Chlordecone (Kepone)
Biochanin A	Dieldrin
β-Sitosterol	Alkylphenols
O-Desmethylangolensin	4-Nonylphenol
Isoflavans	4-Octylphenol
Equol	4-Butylphenol
Coumestans	4-Nonylphenol-diethoxylate (NP2EO)
Coumestrol	4-Nonylphenoxycarboxylic acid (NP1EC)
Lignans	Synthetic Estrogens
Enterlactone	Ethinyl estradiol
Enterodiol	Diethylstilbestrol (DES)
Mycoestrogens	Hexestrol
Zearalenone	
Zearalenol	
Zearalanol (zeranol)	

Steroidal and nonsteroidal synthetic derivatives of natural human estrogens have been developed that have increased the potency, compared to endogenous estrogens,

following oral administration (Bennink and Boerrigater, 2003). Diethylstilbestrol was one of the first non-steroidal estrogens to be synthesized (Figure 2) (Bennink and Boerrigater, 2003). Ethinyl estradiol is an important example of a synthetic derivative. It is a major component of birth control pills. Estrogens are very well absorbed through the skin (Bennink and Boerrigater, 2003). This has allowed the development of transdermal birth control patches.

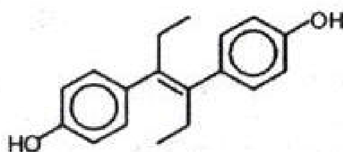


Figure 2: Structure of the synthetic estrogen diethylstilbestrol.

Pharmaceutical estrogens are a source of exogenous estrogens in the environment. Pharmaceutical drugs, including diethylstilbestrol (DES) and oral contraceptives, can be found in raw sewage and in river water (Burton and Wells, 2002; Desbrow et al., 1998). Even after treatment at modern wastewater treatment plants, these estrogenic chemicals can still be detected at low concentrations in the effluent of the plants (Shore et al., 1993).

Industrial chemicals may also have estrogenic effects. Bisphenol A is an essential component of the epoxy resins that are used in metal food cans and has weak estrogenic activity (Dechaud et al., 1999). It can be released from the coatings of these cans and may have an estrogenic effect on the animal consuming the food from the can (Brotons et al., 1995). Bisphenol A is also a component of some sealants and resins used in dentistry and has been shown to be able to leach from them (Olea et al., 1996).

Nonylphenol is used as an antioxidant used in the manufacturing of plastics and has estrogenic activity (Soto et al., 1991). It is known to leach from PVC tubing used in

milk processing and from the packaging of food items (Sonnenschien and Soto, 1998). Nonylphenol has been shown to have weak estrogenic activity.

ESTROGEN ACTION

The plasma half-life of estradiol is very short, just minutes (Bennink and Boerrigater, 2003). To help extend this half-life, animals have an estrogen binding protein in the plasma. This binding protein is sex hormone binding globulin (SHBG). The major roles of SHBG are to act as a buffer or reservoir for active hormones in the plasma and act as a plasma transport system (Damassa et al., 1991). SHBG releases the protein-bound estrogen into the plasma in equilibrium with the free concentration in the animal (Goldstein and Sites, 2002).

All sex steroids require binding to a specific receptor in target tissues to exert an effect (Goldstein and Sites, 2002). They diffuse in and out of all cells in the body, but are retained with high affinity and specificity in target cells. These target cells contain intra-nuclear binding proteins known as estrogen receptors (ERs) (Kuiper et al., 1997; Saunders, 1998; Goldstein and Sites, 2002; Matthews et al., 2000).

Once estrogen is bound by its receptor, the receptor undergoes a conformational change. The dimerization of estrogen receptors is necessary for their functional activity. Estrogen binding to its receptor allows the receptor to bind with high affinity to chromatin and to modulate the transcription of target genes. The steroid hormone and receptor complex then interact with short discrete nucleotide sequences to modulate gene expression. They can interact with these sequences near or sometimes at considerable distances away from the receptor (Cato and Ponta, 1989).

Estrogen Receptors

Estrogen receptors play important regulatory roles in the reproductive, skeletal, and cardiovascular systems and are validated therapeutic targets for diseases such as breast cancer and osteoporosis (Weatherman et al., 2001). Estrogen receptors in different animals have been shown to have different structures. The estrogen receptors from human, mouse, chicken, reptiles, pig, and fish exhibit differential preferences for ligands and relative binding affinities for many natural and synthetic compounds (Matthews et al., 2000). One example of these differential preferences is that the pig estrogen receptor exhibits a significantly greater affinity for the estrogenic mycotoxin zearalenol than does the estrogen receptor from the chicken (Fitzpatrick et al., 1989).

The estrogen, glucocorticoid, and protesterone receptors belong to a large superfamily of ligand-dependent transcription factors (Evans, 1988; Green and Chambon, 1988; Mangelsdorf et al., 1995; Green et al., 1986). This superfamily of ligand-dependent transcription factors possess modular structures composed of a cysteine-rich DNA-binding domain in the middle portion of the receptors. They also contain a carboxy-terminal hormone-binding domain. The amino-terminal ends of the various receptors in this family can vary extensively in both content and size (Cato and Ponta, 1989).

Estrogen receptors are modular in structure and consist of six distinct domains (A-F) (Evans, 1988). A typical estrogen receptor contains a hypervariable N-terminal region that is called the activation function 1 (AF-1) that contributes to the transactivation function. They have a conserved DNA binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain called activation function 2 (AF-2) (Azuma et al., 2004).

Both the AF-1 and AF-2 domains of estrogen receptor alpha are shown to have a transcriptional activation function (Tora et al., 1989). They both also interact with transcriptional mediators and co-factors (Endoh et al., 1999; Rachez et al., 1999; Belandia et al., 2002; Yanagisawa et al., 2002; Belandia and Parker, 2003; Fernandes et al., 2003). The estrogen receptor mediates transcription through binding to estrogen response elements in the upstream promoter regions of target genes (Weatherman et al., 2001). The dimerization function of AF-2 is very important since dimerization is a prerequisite for functional activity of the estrogen receptors (Cato and Ponta, 1989).

Estrogen receptors have been shown to bind several structurally diverse chemicals (Matthews et al., 2000). The ability to bind many different chemicals has been attributed to the size of the ligand-binding pocket. The pocket is almost twice the volume of estradiol (Brozozowski et al., 1997; Kuiper et al., 1998c). Estrogen binding is achieved by a combination of specific hydrogen bonding interactions and the hydrophobic nature of the binding pocket (Matthews et al., 2000). Ligand binding by the estrogen receptor is merely the first step in a pathway that leads to transcriptional activation (Weatherman et al., 2001).

There are two known types of estrogen receptors in mammals and birds. The two N-terminal domains of these types are similar. The DNA binding domains share 96% identity and the ligand binding domains are 50-60% identical (Harris et al., 2002). Radio-ligand binding studies suggest that both estrogen receptors bind equally well to estradiol (Kuiper et al., 1997). It is presumed that estradiol is the natural ligand for both receptors. Although these types of estrogen receptors are so similar, the ability of a compound to selectively bind to a particular estrogen receptor subtype can be species dependent (Harris et al., 2002).

Estrogen Receptor Alpha

Estrogen receptor alpha is generally referred to as the classical estrogen receptor since it was the first to be identified. It is found mainly in the uterus and mammary gland (Gustafsson, 2000). Estrogen receptor alpha is a protein that consists of 595 amino acids with a molecular weight of 66kDa (Kuiper et al., 1996; Furlow et al., 1990; Grohe et al., 1998). Estrogen receptor alpha dimerizes upon the binding of the ligand. The homodimer then binds to estrogen response elements (EREs) in the transcriptional control regions of the target genes (Mosselman et al., 1996).

The role of estrogen receptor alpha in reproductive tissues such as the uterus, and other tissues such as the hypothalamus, pituitary and epididymis has been well-established using knockout mice (Korach et al., 1996; Hess et al., 1997; Hess et al., 2000). These mice have lower reproductive organ weights and often have atrophy of the organs even in the presence of estrogen (Couse and Korach, 1999)

Estrogen Receptor Beta

It was 10 years after the discovery of the original estrogen receptor that a second estrogen receptor was identified (Kuiper et al., 1996). This new receptor was named estrogen receptor beta. It is a protein that consists of 485 amino acids with a molecular weight of 54.2 kDa (Kuiper et al., 1996; Furlow et al., 1990; Grohe et al., 1998). Estrogen receptor beta can bind estradiol and transactivate estrogen regulated reporter genes. It is a less efficient activator of estrogen dependent genes than estrogen receptor

alpha and anti-estrogens can inhibit its effect (Mosselman et al., 1996; Kuiper et al., 1996).

Estrogen receptor beta is found in the uterus and mammary gland (Gustafsson, 2000). It has major importance in tissues of the central nervous, cardiovascular, and immune systems. It is also important in the urogenital tract, bones, kidney, and lung (Enmark et al., 1997; Kuiper et al., 1998a, 1998b, 1997; Gustafsson, 1998).

Estrogen receptor beta is known to play a role in the reproductive functions of female rodents (Hewitt and Korach, 2003). However, the exact role of estrogen receptor beta is still unclear because knockout mice have a relatively mild phenotype (Krege et al., 1998). Estrogen receptor beta knockout mice show signs of prostatic hypertrophy, loss of abdominal fat (lipidystrophy), and possibly an altered bone and skin phenotype (Gustafsson, 2000). These mice also show an exaggerated uterine responsiveness to estrogens, such as edema and increased rates of cellular proliferation in response to estrogen (Weihua et al., 2000).

Estrogen Receptor Gamma

Recently, a novel estrogen binding protein was identified in teleost fish (Hawkins et al., 2000). This binding protein is immunochemically, structurally, and functionally distinct from the two classical steroid hormone receptors (Rao, 1998). It had been previously discovered in three other teleost species, but was not recognized as a distinct estrogen receptor (Hawkins et al., 2000). There are now three major isoforms of estrogen receptors: ER alpha, ER beta, and ER gamma.

Analysis showed that estrogen receptor gamma resulted from a duplication of estrogen receptor beta early in the teleost lineage (Hawkins et al., 2000). There are 22 diagnostic amino acids identified in estrogen receptor gamma. They are located in regions known to be important to receptor-ligand interactions, receptor transactivation, and dimerization (Hawkins et al., 2000).

Functions of Estrogen in Animals

Estrogens contain the cylopentaneperhydrophenanthrene chemical ring structure (Korach et al., 1997). It is this phenolic ring structure that directs binding to specific estrogen receptors and is necessary for estrogen-like activity (Korach et al., 1997). They are formed by the aromatization of androgens. The aromatase enzymes are found primarily in the gonads, but can also be found in the placenta, brain, and adipose tissue (Korach et al., 1997).

Estrogens have numerous physiological actions (Norris, 1985; van Tienhoven, 1983; Korach et al., 1997; Bennink and Boerrigter, 2003). Estrogen influences growth, development, behavior and regulation of reproductive tissues in all vertebrates (Matthews et al., 2000; Zava and Duwe, 1997; Peterson and Barnes, 1996). Estrogens are involved in the development and function of the male and female genital tract (Sonenschein and Soto, 1998) and development of female secondary sexual characteristics (Norris, 1985; van Tienhoven, 1983). Estrogen promotes the hypertrophy of the female secondary sexual organs (Sonenschein and Soto, 1998) and is involved in sexual differentiation of the nervous system (Norris, 1985; van Tienhoven, 1983).

Estrogen is involved in the development and function of breast (Sonenschein and Soto, 1998; Jordan, 1996; Azuma et al., 2004). It has a well-established tumor-promoting

agent that plays a significant role in promoting the growth of estrogen-dependent breast tumors (Zava and Duwe, 1997; Peterson and Barnes, 1996). It is also involved in the development and function of bone (Sonnenschein and Soto, 1998; Azuma et al., 2004) and of neuroendocrine tissues (Sonenschein and Soto, 1998). Estrogen has been shown to help protect against the loss of bone mineral density and cognitive function in post-menopausal women (Jordan, 1996; Howell and Johnston, 2002; Sherwin, 2002). It has also been shown to promote cellular proliferation (Sonenschein and Soto, 1998). It influences regulate circulating cholesterol levels, and has an apparent protective effect on the vascular system in females (Jordan, 1996) and is involved in vascular dilation (Azuma et al., 2004).

PHYTOESTROGENS

Estrogenic compounds that in plants are refered to as “phytoestrogens”. These plant chemicals resemble natural estrogens in either their structure or function (Whitten and Naftolin, 1998; Bradbury and White, 1954). The term phytoestrogen first appeared in the literature in the late 1980’s (Dixon, 2004). Since then, research in this area has increased dramatically. In 1980, there were about 15 scientific papers published on phytoestrogens. In 2002, there were nearly 600 published (Wu et al., 2004).

Phytoestrogens exert their biological effects through receptor and nonreceptor mediated mechanisms. They have the ability to mimic the actions of endogenous

estrogens (act as an estrogen agonist) or inhibit estrogen action (estrogen agonist). Phytoestrogens can also alter the pattern of synthesis and/or metabolism for the endogenous hormones (Sonenschein and Soto, 1998; Whitten et al., 1995).

Sources of Phytoestrogens

Phytoestrogens and other environmental estrogens can come from a wide variety of sources, some of which are consumed by humans and animals (Table 2). An interesting source of estrogenic compounds in humans is coffee (Kaladas and Hughes, 1989). Coffee contains weakly estrogenic components. These estrogenic effects have been shown by increased uterine-to-body weight ratios and total uterine protein content in mice after the administration of coffee extracts (Kitts, 1987).

Table 2: Levels of isoflavones and lignans in various food sources. Values (in nanomoles per gram dry weight) are taken from Mazur (1998). They were determined by isotope dilution gas chromatography mass spectrometry with selected ion monitoring.

Plant species (common name)	Genistein	Daidzein	Secoisolariciresinol	Matairesinol
Soybean	993-3115	413-2205	<1-8	<1
Kidney bean	<1-9	<1-2	2-4	<1
American groundnut	4-30	<1	<1-2	<1
Chickpea	3-8	<1-8	<1	0
Pea	<1	<1	<1	<1
Lentil	<1	<1	<1	<1
Kudzu root	467	7283	<1	<1
Flaxseed	0	0	10,247	30
Sesame seed	<1	6	2	17
Sunflower seed	<1	<1	17	0
Peanut	2	1	8	<1
Wheat bran	<1	<1	3	0
Barley (whole grain)	<1	<1	2	0
Rye bran	0	0	4	5
Strawberry	0	0	33	<1
Cranberry	0	0	29	0
Blueberry	0	0	23	0
Raspberry	0	0	4	0

Red cabbage	<1	<1	4	<1
Broccoli	<1	<1	11	<1
Garlic	0	0	11	<1
Zucchini	0	0	23	<1
Carrot	0	0	10	<1
Beetroot	0	0	3	<1
Black tea	Trace	Trace	73	12
Green tea	Trace	Trace	75	5

Marijuana is also a source phytoestrogens for humans (Kaladas and Hughes, 1989). It has been shown that smoking marijuana can have significant effects on the human menstrual cycle. It can suppress luteinizing hormone levels and shorten both the menstrual cycle and the luteal phase of the cycle (Mendelson et al., 1986). Apigenin is a derivative of flavonoid phytoestrogens found in marijuana and is a relatively potent inhibitor of estradiol binding to uterine estrogen receptors (Sauer et al., 1983). Tetrahydrocannabinol (THC), the psychoactive chemical found in marijuana, is not specifically considered to be an estrogen agonist, but it does produce estrogen-like effects. These effects may be produced through neuroendocrine pathways (Smith et al., 1979).

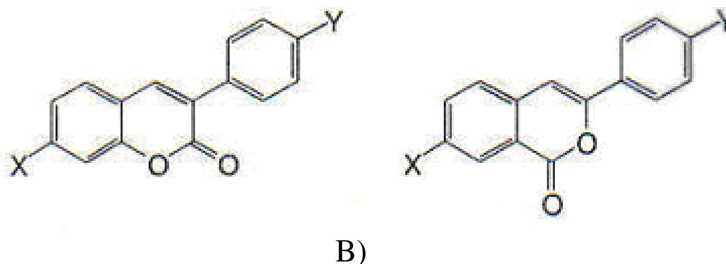
Environmental contaminants of plant origin, concentrated and released by human activity, are though to have estrogenic effects on animals. Fish species have shown to be affected by the estrogenic effects of the phytoestrogen β-sitosterol. β-sitosterol has a four ring structure that is very similar to cholesterol and the vertebrate reproductive steroids such as estradiol (MacLatchy and Van Der Kraak, 1995). It is found in high concentrations in plant oils, legumes and in wood (Pollack and Kritchevsky, 1981). Because it is present in wood, it is released in high concentrations in the effluent discharged from bleached kraft pulp and paper mills (MacLatchy and Van Der Kraak, 1995) into surface waters where it causes estrogenic effects on aquatic organisms.

Many phytoestrogens have been isolated from various types of alcoholic beverages including bourbon and beer. These have been shown to contain β-sitosterol, biochanin A, genistein, and daidzein. All of these chemicals have the ability to bind to estrogen receptors and induce biological responses (Gavaler et al., 1995; Rosenblum et al., 1993). Estrogenic activity has also been detected in wines (Gavaler et al., 1995). Red wines have been shown to have a high content of phenolic substances. These include catechin and resveratrol. Resveratrol is a stilbene and has been shown to be an estrogen agonist (Ratna, 2002; Bowers et al., 2000).

Classes of Phytoestrogens

Coumestans

Phytoestrogens are broken down into three major classes, which are coumestans, lignans, and isoflavones. Coumestans contain central structures of 15 carbons (Figure 3) (Kaldas and Hughes, 1989). Coumestrol is the major phytoestrogen in this class. It has the ability to function as an antiestrogen in the brain of animals (Patisaul et al., 1999) and has been shown to have strong estrogenicity similar to estradiol in rat uterotrophic assays (Tinwell, 2000).



A) B)

Figure 3: Structures of coumestans: A) Coumarin, and B) Isocoumarin.

Coumestans mainly occur in alfalfa, clover and annual medics (Dixon, 2004), particularly in plants that are suffering from a foliar disease (Adams, 1995). Coumestrol is the most potent of all the phytoestrogens, but is 100-200 times less potent than 17β-estradiol and almost 3000 times less potent than diethylstilbestrol (DES) (Elakivich and Hampton, 1984; Hopert et al., 1998).

Lignans

The major lignans are enterolactone and enterodiol. They are the products of the microbial metabolism of secoisolariciresinol and matairesinol and are formed in the gut (Dixon, 2004; Setchell et al., 2002; Wang, 2002). Secoisolariciresinol and matairesinol are components of whole grains, fibers, flax seeds, and several different fruits and vegetables (Barrett, 1996). Enterolactone does not accumulate in plants, but is derived from the further oxidation of enteroldiol (Barrett, 1996).

Isoflavones

The isoflavonoid class, which contains isoflavones and isoflavans, is the largest class of phytoestrogens (Korach et al., 1997). Isoflavones are low molecular weight diphenolic compounds (Burton and Wells, 2002) that are derived from flavones. Flavones are compounds that are present in almost all plant families and can be isolated from most plant tissues including the leaves, stems, roots, flowers, and seeds (Verdeal and Ryan, 1979; Harborne, 1971).

Isoflavones are the monocarboxylic derivatives of the 15 carbon flavones (Kaladas and Hughes, 1989) and differ from flavonoids by having the B-ring linked to the 3- rather than the 2- position on the central heterocycle (Dixon, 2004). The exact

position and number of the hydroxyl substituents on the isoflavone molecule seems to determine the estrogen receptor binding affinity (Kuiper et al., 1998c).

Isoflavones are mostly limited to the subfamily Papilonoideae of Leguminosae (Dewick, 1994). They are often antimicrobial compounds (phytoalexins) that are synthesized de novo in response to the plant's exposure to a pathogen (Burton and Wells, 2002). Most phytoestrogens display their hormone-like activity over a concentration range of 0.1 to 10 um (Miksicek, 1994, 1993).

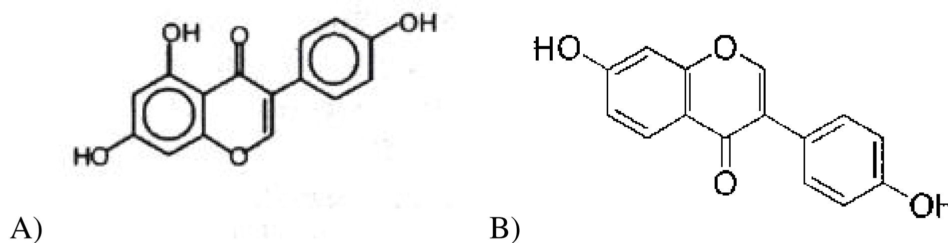


Figure 4: Structures of isoflavones: A) Genistein and B) Daidzein.

The four most common isoflavones are formononetin, daidzein, genistein, and biochanin A (Figure 4) (Barrett, 1996, Thomas 1997). Isoflavones are each found in four chemical forms (Table 3). The unconjugated forms or aglycones include daidzein, genistein, and glycitein (Kudou et al., 1991). The glucoside form is a plant sugar derivative (Barrett, 1996). These include daidzin, genistin, and glycitin. There are also acetylglucosides and malonylglucosides (Kudou et al., 1991). The aglycones may be more bioavailable than glucosides (Kurzer and Xu, 1997). The O-methylation of isoflavones decreases their estrogenicity in *in vitro* ER binding assays. Because of this, formononetin and biochanin A are less potent than daidzein and genistein (Dixon, 2004).

Table 3: Various forms of isoflavones.

Aglycones	Glucoside	Acetylglucoside	Malonylglucoside	4'-methyl ethers
Daidzein	Daidzin	6"-O-acetyldaidzin	6"-O-malonyldaidzin	Formononetin

Genistein	Genistin	6"-O-acetylgenistin	6"-O-malonylgenistin	Biochanin A
Glycitein	Glycitin	6"-O-acetylglycitin	6"-O-malonylglycitin	

Soybean Phytoestrogens

The production of soybeans in the 2002-2003 year was estimated to be 197 million metric tons worldwide. Of these, 74.8 million metric tons were produced in the United States (Agricultural Statisitics, 2004). Most soybeans are processed into soybean meal and oil. In 2001, the poultry industry consumed nearly half of the soy meal that was produced in the United States (American Soybean Association, 2003). Soy food for both human and animal consumption contains large amounts of phytoestrogens (Anderson and Wolf, 1995; Aussenac et al., 1998).

The concentration of isoflavones in plants can vary depending on different types of stress, including viral, bacterial, fungal, and herbivore attacks (Barrett, 1996). Isoflavones are associated with the response of soybeans to infection by *Phytophthora megasperma* (Graham et al., 1990). Isoflavones can also be important signals to attract the *Rhizobia* bacteria that form the nitrogen-fixing nodules on the roots of soybean plants (Phillips, 1992), and they can also induce *nod* genes in the bacteria (Cho and Harper, 1991; Kape et al., 1991; Smit et al., 1992).

Isoflavone concentrations in soybeans can vary due to climate and soil conditions (Franke et al., 1994). The concentrations of isoflavones were found to increase in soybeans that are produced on low- to medium-potassium soils (Wu et al., 2004). Soybean seeds that mature at low temperatures were shown to have greater isoflavone concentrations than those seeds that matured at high temperatures (Lee et al., 2003; Tsukamoto et al., 1995).

Nearly 90% of the total isoflavones found in the soybean are located in the cotyledon. The rest of the isoflavones are located in the hypocotyl (Tsukamoto et al., 1995). Isoflavones can constitute about 0.3 to 0.8% of the soybean seed on a dry weight basis (Lee et al., 2003). Some soy products can contain up to 6000 ppm genistein or up to 8000 ppm of total phytoestrogens when daidzein is also considered (Mambrini et al., 1999).

Genistein, daidzein and glycitein are the major isoflavones found in soybeans and are generally found at a ratio of approximately 1.3:1.0:0.2 respectively (LC Laboratories, 2004). Unprocessed soybeans contain 1.2 – 4.2 mg of isoflavones/g dry weight (Kurzer et al., 1997), which can range from 0.14-1.53 mg/g.

The variation in the isoflavone content of soybeans is evident in the isoflavone content of various soy products made from different crop years and varieties of soybeans (Song et al., 1998; Anthony et al., 1996; Barnes et al., 1994). Malonylglucosides are the predominant form of isoflavone found in soybeans. The concentrations of the glucoside, acetylglucoside, and aglycone forms tend to increase during extraction, processing and cooking (Wang and Murphy, 1994; Coward et al., 1998; Eldridge and Kwolek, 1983; Kurzer and Xu, 1997). Heat processing, enzymatic hydrolysis, and fermentation can significantly alter the form of isoflavones in soybeans by reducing the malonylglucosides and increasing the glucosides and acetylglucosides (Wang and Murphy, 1996; Song et al., 1998). Glucosides make up to 50-98% of the isoflavones that are found in soy foods. Only extensively fermented foods such as tempeh have higher concentrations of aglycones than glucosides (Song et al., 1998).

In commercial practices, defatted soybean meals will contain essentially all of the isoflavone glucosides that are present in the starting soybeans (Eldridge and Kwolek,

1983). Soy concentrates can be produced with an ethanol-washing step that results in a product that has almost no isoflavones (Song et al., 1998).

Soybean isoflavones possess many biological activities that seem to support the health of normal cells. They also encourage apoptosis in diseased cells (McCue and Shetty, 2004). It is possible that the cancer-preventative effects of soy are due to other components. These may include saponins, protease inhibitors, phytic acid, phytosterols, or phenolic acids (Messina and Messina, 1991).

General Effects on Animals

The relative estrogenic potency of an environmental estrogen is dependent upon many things. These include animal species, dosage, and route of administration. The duration and timing of exposure (Whitten and Patisaul, 2001), specific target tissue and the functional state of this tissue can also affect the relative potency (Kaldas and Hughes, 1989).

The timing of exposure during the life cycle of the animal greatly affects the severity of the biological response (Bigsby et al., 1999). Exposure to environmental estrogens during development and early life can have marked effects on the reproductive system that may persist through the lifetime of the animal (McLachlan, 1985; Colburn and Clement, 1992). Early development is a period of increased sensitivity to both endogenous and exogenous estrogens that can affect the development of the brain and reproductive tract (Whitten and Naftolin, 1994; Toppari, 1996).

Differentiation and development of the gonads are under complex regulation from hormones and growth factors. Environmental endocrine disrupters may impose greater negative effects on gonadal development early in development. Exposure to these

chemicals during embryonic development is likely to cause long-term reproductive impairment in many mammals and precocial birds, possibly even leading to population-level effects (Ottinger et al., 2001). A low dose phytoestrogen diet can induce developmental and maturational abnormalities in both laboratory animals and domestic livestock (Burton and Wells, 2002). The effects of phytoestrogens on livestock and laboratory species have been studied extensively, but field biology studies in many wild herbivore species are lacking (Hughes, 1988b).

The postnatal period is possibly the most sensitive period to dietary influences on sexual development (Odum et al., 2001). Abnormal reproduction system development, both structural and functional, could lie latent until adolescence or adulthood (Naciff et al., 2002). Breast and uterine cancer, endometriosis and altered development of the brain and reproductive tract can be effects of exposure to exogenous estrogens (Whitten and Naftolin, 1994; Toppari et al., 1996; Crisp et al., 1998).

The ingestion of phytoestrogens stimulates the hepatic synthesis of sex hormone binding globulin (SHBG) and indirectly reduces the amount of free (biologically active) estradiol in the serum (Adlercreutz et al., 1987). Estradiol that is bound to estrogen receptors or SHBG can be displaced by phytoestrogens. A large dose of phytoestrogens will displace more estradiol than a small dose (Verdeal et al., 1980).

Enterolactone, genistein, and daidzein have been shown to stimulate SHBG synthesis in cultured hepatocytes (Adlercreutz and Mazur, 1997). The binding of these xenoestrogens to SHBG does not prevent the reassociation of natural SHBG ligands (Dechaud et al., 1999). Xenoestrogens binding to SHBG may affect the metabolism and tissue bioavailability of these chemicals (Damassa et al., 1991). The xenoestrogens could

displace estradiol from the SHBG binding sites and may enhance the estrogen amplification effect of SHBG (Burke and Anderson, 1972).

Isoflavones have also been shown to exert effects on thyroid hormones, cortisol, and insulin (Duncan et al., 1999). In the absence of estrogen, isoflavones show a weakly estrogenic effect. They may exhibit an estrogenic or anti-estrogenic effect when estrogen is present (Cline and Hughes, 1998). Indirect effects of phytoestrogens could also occur through the augmentation of suppression of estrogen negative feedback in the anterior pituitary or hypothalamus (Whitten and Naftolin, 1998). The actions of phytoestrogens would depend on the concentration and potency. Prolifertive actions would be more probable than suppressive actions for the more potent soy isoflavones such as genistein and daidzein (Whitten and Naftolin, 1998).

Rats that consume soy in place of other proteins generally develop 25-50% fewer tumors than control animals (Kurzer, 2003). Animal studies suggest that dietary isoflavones may also exert benefits on bone mineral density (BMD) and bone turnover in ovariectomized rats (Ishida et al., 1998; Fanti et al., 1998).

GENISTEIN

Genistein is biosynthetically the simplest of the isoflavonoid compounds of the leguminosae and is a central intermediate in the biosynthesis of more complex isoflavonoids (Dixon and Ferreira, 2002). Genistein is 4’, 5, 7-trihydroxyisoflavone, a diphenolic planar molecule with an aromatic A-ring (Barnes and Peterson, 1995). Like estrogen, it has a second oxygen atom that is 11.5 Å from the one in the A-ring and a molecular weight that is similar to those of steroidal estrogens (Lamartiniere et al., 1995).

Genistein and daidzein, the two most prevalent isoflavones in the soybean, are very similar, but differ in a few important ways. Genistein has an additional 5-hydroxy group as compared to daidzein (Barnes and Peterson, 1995). It is also more hydrophobic than daidzein. This is because genistein has hydrogen bonding of the 5-hydroxy group with the 4-ketonic oxygen (Barnes and Peterson, 1995). Genistein and daidzein both contain the phenolic ring structure that is a key characteristic found in the majority of the ligands that bind the estrogen receptors (Setchell, 1998).

Genistein attains a higher plasma concentration than daidzein when administered at the same level. However, because of its greater hydrophobicity it is not as widely distributed within the animal. Genistein also has a greater bioavailability than daidzein. The overall bioavailability of both compounds may increase if the compounds are ingested as their glycosides (Setchell et al., 2001).

Metabolism of Genistein

In the animal gut, the naturally occurring glycosides are hydrolyzed into aglylcones (Barrett, 1996; Day et al., 2000). The aglycones and their metabolites are then excreted, absorbed into the blood through the intestine and metabolized (Barrett, 1996). In the intestine, they are mostly conjugated into glucuronides and/or sulphates prior to their release into the blood stream. They then travel though the blood and get transported to the liver (Wu et al., 2004).

The isoflavones formononetin and biochanin A are metabolized to daidzein and genistein respectively by the normal intestinal flora. These new forms are more potent estrogens than their plant precursors (Korach et al., 1997; Barrett, 1996; Dixon, 2004). If

genistein is not absorbed into the body, it is bacterially metabolized into p-ethylphenol, which is a hormonally inert compound (Barrett, 1996).

Metabolism of genistein has been characterized in both the rat and man. These metabolites include genistein glucoronide, dihydrogenistein glucuronide, genistein sulphate, dihydrogenistein, and 6'-hydroxy-O-desmethylangolensin (Coldham et al., 1999). Genistein is naturally deglycosylated and absorbed into the blood stream as 7-O-β-glucuronide, not as genistein (Bennetau-Pelissero et al., 2001; Sfakianos et al., 1997; Yuan et al., 2003). Both genistein and 7-O-β-glucuronide are not very well absorbed from the intestine. Also, they are very efficiently extracted from the portal blood in the liver and then excreted into the bile in a conjugated form (sulfate ester or glucuronide) (Sfakianos et al., 1997). Due to this efficient enterohepatic circulation, genistein may accumulate within the enterohepatic circuit or may be excreted with a long half-life (Sfakianos et al., 1997).

The renal excretion of genistein after ingestion has been used to evaluate its bioavailability (Xu et al. 1994). Researchers have determined that genistein is poorly bioavailable because 3-10% of the dose appears in the urine. This approach does not take into account the amount of genistein or its metabolites that may be excreted through the feces (Sfakianos et al., 1997). The bioavailability, lipophobicity, metabolism, and pharmacokinetics of environmental compounds must be considered when evaluating their estrogenic potency (Korach et al., 1997).

Analytical Methods to Determine Genistein Levels

The effects of phytoestrogens are clearly linked to the ingested doses and ingested amounts can be found in biological fluids. Quantatative analysis of soy isoflavones in biological fluids can be accomplished with a variety of methods and analytical instrumentation. Genistein can be determined in biological fluids by gas chromatography-mass spectrometry (GC-MS) or by high performance liquid chromatography (HPLC) (Adlercreutz et al., 1994; Joannou et al., 1995; Franke and Custer, 1996). HPLC with UV detection is one of the simplest methods to determine isoflavone levels in both plant extracts and animal tissues or body fluids (Dixon, 2004; Liu et al., 2002; Wang, 2002). It has relatively easy operation and low cost as compared to LC-MS. Recently an HPLC-UV method for the quantification of genistein, daidzein, glycitein, and their primary metabolites in human plasma and urine was created. This method was validated with the US FDA guidelines for the validation of methods used in pharmokinetic studies (Thomas et al., 2001).

Combined liquid chromatographic and mass spectrometric techniques (LC-MS) have been contributing to the progress of phytoestrogen analysis (Wu et al., 2004). There has not yet been a standardized assay method developed for phytoestrogens. This means that the concentrations listed in the soy-based supplements should be regarded with caution (Bennetau-Pelissero et al., 2003).

The commonly used immunoassay types for the analysis of phytoestrogens include radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and time-resolved fluoroimmunoassay (TR-FIA) (Wu et al., 2004). ELISA is an inexpensive, usually rapid and sensitive test that may not require complex extraction steps or methods. It can often be used for large-scale studies including surveys and epidemiological studies

(Bennetau-Pelissero et al., 2003). It is based on the use of specific antibodies directed against the phytoestrogen. Currently, only genistein, daidzein, and equol can be measured with this technique. Because of this, ELISA is only relevant to assay to plants producing these compounds or biological fluids from animals consuming these plants (Bennetau-Pelissero et al., 2003).

Microarray technology is now being adapted for use in toxicology research. This use of microarray technology has been termed toxicogenomics (Nuwaysir et al., 1999; Pennie et al., 2000; Rodi et al., 1999). The premise of the toxicogenomics is that identifying the gene-expression profiles induced by the different toxicants should result in a distinct "molecular fingerprint" that is representative of the specific toxicant (Naciff et al., 2002). However, this has not been validated forgenistein orother phytoestrogens.

Effects of Genistein

Molecular Effects

All isoflavones studied so far have antimicrobial activity. Genistein may function as both a phytoalexin (inducible) and a phytoanticipin (pre-formed) antimicrobial (Dixon and Ferreira, 2002). Genistein and daidzein are strong antioxidants (Peterson and Barnes, 1991; Barnes, 1998; Kurzer and Xu, 1997; Yen and Lai, 2003; Rimbach et al., 2003; Barnes and Peterson, 1995; Peterson, 1995; Barnes et al., 1994). They account for 80% of the antioxidant potential of soybeans (Hsu et al., 2001; Hwang et al., 2001; Arora et al., 2000).

Genistein has been shown to inhibit protein tyrosine kinases (PTK) that are central components in the signal transduction process (Barrett, 1996; Osada et al., 1988; Akiyama et al., 1987; Messina et al., 1994; Peterson and Barnes, 1991; Barnes, 1998;

Kurzer and Xu, 1997; Watanabe et al., 1991; Brown et al., 1998; Chapin et al., 1996; Makarevich et al., 1997; Barnes and Peterson, 1995; Peterson, 1995; Barnes et al., 1995). It can also inhibit protein kinase A (PKA) (Makarevich et al., 1997) and protein kinase C (PKC) (Osada et al., 1988; Akiyama et al., 1987).

Genistein can decrease calcium-channel activity in neurons (Potier and Rovira, 1999). It decreases lipid peroxidation (Arora et al., 1998) and diacyglycerol synthesis (Dean et al., 1989). Genistein can also inhibit DNA topoisomerases (Barrett, 1996; Messina et al., 1994; Akiyama et al., 1987; Okura et al., 1988; Barnes and Peterson, 1995; Peterson, 1995; Barnes et al., 1994).

Genistein can inhibit angiogenesis in *in vitro* experiments (Barrett, 1996; Barnes and Peterson, 1995; Peterson, 1995; Barnes et al., 1994) and inhibit cell cycle progression (Barnes and Peterson, 1995; Peterson, 1995; Barnes et al., 1994). It has also been shown to inhibit nitric oxide synthase (Duarte et al., 1997). It has been found to inhibit aromatase, which would have significant reproduction effects through inhibition of estrogen synthesis (Peterson and Barnes, 1991; Barnes, 1998; Kurzer and Xu, 1997). Genistein has radical-scavenging activity (Yen and Lai, 2003; Rimbach et al., 2003) and has also been shown to be a specific inhibitor of GABA receptors in rats (Huang et al., 1999)

The anticancer function of soybeans has been associated with genistein (Messina et al., 1994). Genistein promotes the growth and development of neoplasia in several target organs (Peterson and Barnes, 1993; Rokhlin and Cohen, 1995; Peterson and Barnes, 1996). It can induce apoptosis in cancer cells (Spinozzi et al., 1994; Constantinou et al., 1998; Katdare et al., 2002). It also suppresses the expression of

specific oncogenes (Okura et al., 1988; Zwiller et al., 1991; Yamashita et al., 1990; Linassier et al., 1990).

Genistein inhibits tumor cell growth and induces cellular differentiation of some malignant cells (Okura et al., 1988; Zwiller et al., 1991; Yamashita et al., 1990; Linassier et al., 1990). It also modulates tissue differentiation and cellular proliferation (Peterson and Barnes, 1993; Rokhlin and Cohen, 1995; Peterson and Barnes, 1996). Unlike other isoflavones, genistein only exerts toxicity at concentrations far greater than those needed for biological and pharmacological effects (Dixon and Ferreira, 2002). This makes it a potentially important molecule for dietary cancer chemoprevention.

General Effects on Animals

Genistein shares structural features with the potent estrogen 17-β estradiol. It is particularly similar in the phenolic ring and the distance between its 4'-and 7'- hydroxyl groups (Dixon and Ferreira, 2002). These features allow genistein to bind to estrogen receptors in animals (Hopert et al., 1998; Milligan et al., 1998; Messina et al., 1994; Makarevich et al., 1997). The ligand:receptor complex it creates induces the expression of estrogen-responsive genes that can result in an increased uterine mass (Santell et al., 1997; Hopert et al., 1998; Milligan et al., 1998). When genistein binds to receptors, it can also alter the affinity of the estrogen receptor complex for various estrogen response elements (EREs) (Nikov et al., 2000).

Genistein has been shown to bind preferentially to estrogen receptor beta as compared to binding to estrogen receptor alpha (Windahl et al., 2000; An et al., 2001; Morito et al., 2001; Nikov et al., 2000). It binds to estrogen receptor beta with about 25 fold higher affinity than to estrogen receptor alpha (Kuiper et al., 1998c). Genistein

increases the synthesis of sex hormone binding globulin (SHBG) (Peterson and Barnes, 1991; Barnes, 1998; Kurzer and Xu, 1997; Pino et al., 2000) and can displace bound estrogen and testosterone from SHBG (Pino et al., 2000). Genistein also has the ability to prevent the full release of pituitary gonadotropin (GTH) (Hughes, 1988a) and thus can act on the sexual differentiation of mammals (Levy et al., 1995).

Genistein can function as both an estrogen agonist and antagonist in animals (Adlercreutz, 1990). Its effect on animals seems to be based on the ratio of estradiol to genistein at the target organ (Adlercreutz et al., 1995). When injected in high doses, genistein treatment results in alterations to the ovarian steroid hormone production. This is likely to occur through perturbations in signaling via the hypothalamic-pituitary-ovarian axis and through inhibiting aromatase (Cotroneo et al., 2001). Effects of prenatal genistein are different from those associated with neonatal treatment with genistein. This suggests that timing of exposure or maternal and placental influences are important in development of these characteristics (Levy et al., 1995).

Effects on Birds and Poultry

Estrogen is a pivotal factor involved in the development of sexual differentiation, female secondary sexual characteristics, and vitellogenesis in birds (van Tienhoven, 1983). The embryonic genital tubercle, gonads, and Mullerian ducts all have the capacity to respond to estrogens (Andrews et al., 1997).

Embryonic exposure to a potent endocrine disrupting chemical (EDC) is likely to have both short-term effects on neuroendocrine and endocrine impacts as observed at hatch as well as long-term effects that will persist in the maturing and adult animal (Ottinger et al., 2005). Embryonic estrogen exposure results in abnormal oviducts in

adult quail and domestic fowl (Greenwood and Blyth, 1938; Rissman et al., 1984; Holm et al., 2001). These abnormalities include retention of the right-side oviduct, reduced size of the left-side oviduct, impaired egg laying, disrupted distribution of carbonic anhydrase in the shell gland, and production of shell-less eggs (Greenwood and Blyth, 1938; Rissman et al., 1984; Holm et al., 2001).

The Japanese quail and other avian species are common models animal for studying long-term effects of embryonic exposure to estrogenic compounds (Halldin, 2005). Treatment of quail embryos with estradiol alters adult reproductive performance (Whittsett et al., 1997). Reproductive success in the California quail was negatively correlated with the presence of phytoestrogens in forage plants. During dry years when food supplies were low, many forages contained high levels of phytoestrogens. The egg production and hatching success at this time was decreased (Leopold et al., 1976).

In Japanese quail, estrogens produced by aromatization of testosterone in the brain activate the male copulatory behavior and also regulate the concentration of aromatase and of its messenger RNA (Balthazart and Foidart, 1993; Balthazart, 1997; Balthazart and Ball, 1998). Male Japanese quail treated embryonically with estradiol show neither normal copulatory behavior nor normal vasotocinergic innervation in dimorphic brain regions (Panzica et al., 1998). Chickens have been reported to be more sensitive than other avian species to multiple toxicants (Scanes and McNabb, 2003).

It has been shown that the blood plasma of hens fed an isoflavone-enriched diet contain mostly conjugated forms of isoflavones and less of the glycoside and aglycone forms (Saitoh et al., 2004). This suggests that dietary soybean isoflavone-glycosides were deglycosylated in the hen’s intestinal tract and absorbed as aglycones, which were then

metabolized and conjugated in intestinal epithelial cells and released in the bloodstream as conjugated forms (Saitoh et al., 2004).

Genistein found in the diets of birds consuming soybean meal can be transferred into the yolk of eggs produced by those birds (Lin et al., 2004). The levels of yolk steroids in eggs reflect the circulating concentration in the female at time yolk was laid down (Dawson, 2000). Genistein is detectable in the egg yolks of both genstein- and genistin- treated quail and is transferred more efficiently into the eggs as compared to other isoflavones such as daidzein (Lin et al., 2004).

Researchers have found that most of the isoflavones in the blood plasma were present in the high-density fraction, but not the low-density lipoprotein-rich fraction (Saitoh et al., 2004). The presence of isoflavones in more hydrophilic forms, such as conjugates, than aglycones shows that dietary isoflavones form complexes directly with yolk precursor lipoproteins, such as vitellogenin, in the laying hens' blood. They are co-transported as lipoprotein complexes into the oocyte (Saitoh et al., 2004).

Shortly before the final days of ovulation, the majority of yolk components are transported into the growing oocytes from the bloodstream (Nimpf et al., 1991, Adkins-Regan et al., 1995). Some isoflavones, including genistein, can be deposited in the egg (Lin et al., 2004). Aglycone isoflavones would be more effectively absorbed and transferred to the egg yolk than their glycosylated conjugates (Lin et al., 2004; Saitoh et al., 2001). The isoflavone conjugates detected in the egg yolk might be transported from the blood into the growing oocyte, possibly as complexes with yolk precursors.

The specialization of primitive reproductive tissue in response to hormonal stimulation during sexual maturation is one of the few instances in which dramatic cyto-differentiation in vertebrates is not restricted to the periods of embryonic development

(Kohler et al., 1969). Daily administration of estrogen results in a marked increase in the wet weight of the oviduct (Oka and Schimke, 1969). Quail and chickens fed o,p'-DDT showed an increase in oviduct weight similar to those fed a 100-fold less dose of estradiol (Bitman et al., 1968).

The immature chick oviduct affords a unique opportunity to trace the sequential events in the synthesis of protein for export under externally controlled conditions. The increasing proportion of oviduct weight, which is represented as ovalbumin in the course of DES stimulation suggests that a relatively larger proportion of the proteins constituting the actual synthetic machinery must be produced early in differentiation (Kohler et al., 1969). The administration of exogenous estrogen to the immature chick has been shown to result in impressive increases in oviduct size and weight. These are proportional to the amount of hormone given (Hertz and Tulner, 1947; Munro and Kosin, 1943).

The mucosa of the magnum portion of the chick oviduct is a tissue well suited to the study of controlled hormone-induced differentiation. After stimulation with exogenous estrogen, three distinct types of epithelial cells differentiate from the previously indistinguishable immature epithelial cells of the chick oviduct mucosa (Brant and Nalbandov, 1956). Two of the types of chick oviduct epithelial cells that are derived after stimulation by exogenous estrogens synthesize cell-specific proteins, which may be used as markers for differentiation. The tubular glands produce ovalbumin and the goblet cells synthesize avidin (Kohler et al., 1968). The third cell type that is differentiated after stimulation by estrogen is ciliated and is used to propel material through the oviduct (Kohler et al., 1969).

The neonatal chick is known to be very susceptible to estrogen treatment (Munro and Kosin, 1940). Estrogens have a crucial role in the development of both the gonads

and the genitals of chickens (Andrews et al., 1997). In the chicken, as in most avian species, only the left ovary attains functional development. The left testis also develops more strongly than the right (Romannoff, 1960; Marshall, 1961). The slow, natural growth of the chick oviduct ordinarily continues until sexual maturation at about 100 days of age (Brant and Nalbandov, 1956).

Posthatch estrogen treatment is known to alter development of the oviduct anatomy of chicks (Kohler et al., 1969; Oka and Schimke, 1969). Repeated administration of estrogen to the immature female chick results in rapid cell proliferation and formation of tubular gland cells. It also causes specific egg white proteins such as lysozyme and ovalbumin to appear in the oviduct (Oka and Schimke, 1969). The administration of DES induces the synthesis of ovalbumin in the immature chick oviduct (Kohler et al., 1968; O'Malley, 1967). Chickens treated with aromatse inhibitors also lack oviducts, suggesting that estrogens may have a role in Mullerian duct development (Elbrecht and Smith, 1992).

Genistein or its metabolites may function as partial agonists at the chicken estrogen receptor with regard to the stimulation of expression of e-RmRNASF (Ratna, 2002). Genistein, due to its lower potency as compared to estrogen, functions as a partial agonist at the chicken estrogen receptor. At certain ratios, it can block the activity of the full agonist estrogen (Ratna, 2002). It appears that daidzein, unlike genistein, has no effect on the chicken estrogen receptor (Ratna, 2002).

Isoflavones are a potential dietary supplement that may affect lean and fat deposits. Isoflavones may have efficacy as a feed supplement to decrease fat deposition in animals because of this estrogen-like function. At very high concentrations, soy

isoflavones depress the growth rate and gain:feed ratio in commercial broiler type chickens (Payne et al., 2001).

III. STATEMENT OF RESEARCH OBJECTIVES

The objective of the study was to examine the estrogenicity of the soy isoflavone genistein in the immature chicken by observing the potential stimulation of the growth response of the chick oviduct, induction of ovalbumin in the oviduct, the amount of genistein, vitellogenin, and protein present in the plasma, and through the deposition of medullary bone in the femur.

IV. MANUSCRIPT 1

ESTROGENIC EFFECTS OF SHORT-TERM ORAL EXPOSURE TO THE SOY ISOFLAVONE GENISTEIN IN THE IMMATURE BROILER CHICKEN

Abstract

To determine the effects of short-term exposure to the putatively estrogenic soy isoflavone genistein on the oviduct of immature female chickens; 100 chicks were wing banded, individually weighed, and placed in battery brooders. Chicks were assigned to ten treatment groups. Two replicate groups per treatment received a daily gavage of either 0.2 ml corn oil vehicle (CV); 1 mg diethylstilbestrol (DES); 2.0 mg genistein (G2); 20 mg genistein (G20); or 40 mg genistein (G40). All groups were fed a low isoflavone egg-white based chick starter diet. At 15 days of age, one replicate per treatment received a single injection of 2 mg progesterone in a corn oil vehicle to induce ovalbumin synthesis in the oviduct. Oviduct growth in response to estrogen, induction of progesterone receptor and initiation of ovalbumin synthesis was examined by immunohistochemistry. At 16 days of age, DES treatment increased absolute oviduct weight and relative oviduct weight as compared to all other treatments ($P<0.05$). Immunohistochemistry of formalin fixed oviduct samples revealed that the DES, G20, and G40 treatments increased specific staining for progesterone receptor and induced synthesis of ovalbumin in the chick oviduct as compared to CV and G2 treatments. These

results indicate that genistein acts as a weak estrogen with selective effects on the chick oviduct.

Introduction

Phytoestrogens are estrogenic compounds that are found in plants and resemble natural estrogens in either their structure or function (Whitten and Naftolin, 1998; Bradbury and White, 1954). Phytoestrogens fall under the category of "selective estrogen receptor modulators" (SERMs), which are compounds that bind estrogen receptors and produce effects similar to endogenous estrogen (estrogen agonists), inhibit the expression of estrogen action through competition with endogenous hormone for receptor binding, or through blocking or inhibiting receptor actions (estrogen antagonists). Phytoestrogens can also alter the pattern of synthesis and/or metabolism for the endogenous hormones (Sonnenschein and Soto, 1998; Whitten et al., 1995).

The isoflavonoid class, containing isoflavones and isoflavans, is the largest class of phytoestrogens (Korach et al., 1997). Isoflavones are derived from flavones, which are compounds that are present in almost all plant families (Thomas, 1997). Soybeans and soy-based products have been found to be the most significant dietary sources of isoflavones for humans and livestock (Kurzer and Xu, 1997).

Daidzein, genistein, and their glucoside conjugates represent the major soy isoflavones (Wu et al., 2004). Each of these isoflavones is found in four chemical forms. The unconjugated forms, or aglycones, are daidzein, genistein, and glycitein. Each of these isoflavones is also found as a glucoside. These are daidzin, genistin, and glycitin. In clover, the 4'-methyl ethers of daidzein and genistein, formononetin and biochanin A, can be found (Kurzer and Xu, 1997). In commercial practice, defatted soybean meals will

contain essentially all of the isoflavones or isoflavone glycosides present in the starting soybeans (Eldridge and Kwolek, 1983).

Genistein shares structural features with the potent animal estrogen 17-β estradiol. It is particularly similar in the phenolic ring and the distance between its 4'-and 7'-hydroxyl groups. These features allow genistein to bind to estrogen receptors and sex hormone binding proteins (Hopert et al., 1998; Milligan et al., 1998; Messina et al., 1994; Makarevich et al., 1997). This binding allows genistein to displace the natural estrogen and promote its effects.

Immature female rodents and chicks have long been used in bioassays of estrogenicity. Administration of estrogenic compounds to the immature mouse, rat, or chick results in a significant increase in uterine or oviduct mass, as appropriate, proportional to the dose of the estrogen given (Hertz and Tullner, 1947; Munro and Kosin, 1943; Oka and Schimke, 1969). In mammalian studies, genistein has been demonstrated to similarly stimulate uterine weight through an estrogen receptor dependent mechanism (Santell et al., 1997; Hopert et al., 1998; Milligan et al., 1998).

The chick oviduct is a tissue suited to the study of controlled hormone-induced differentiation. After exogenous estrogen stimulation, three distinct types of epithelial cells differentiate from previously indistinguishable immature epithelial cells of the chick oviduct mucosa (Brant and Nalbandov, 1956). These cell types include tubular glands, goblet cells, and a ciliated cell. Repeated administration of estrogen to the immature female chick results in rapid cell proliferation and formation of tubular gland cells. It also causes specific egg white proteins such as lysozyme and ovalbumin to appear in the oviduct under stimulation by progesterone (Oka and Schimke, 1969).

The timing of exposure during the life cycle of the animal greatly affects the severity of the biological response (Bigsby et al., 1999). Exposure to environmental estrogens during development and early life can have marked effects on the reproductive system that may persist through the lifetime of the animal (McLachlan, 1985; Colburn and Clement, 1992). Exposure of the uterus to estrogen in developing pigs has effects on the uterine weight, cell proliferation, and protein expression, which have detrimental effects on adult reproduction (Bartol et al., 1999; Spencer et al., 1993). This research showed that the magnitude of response, reflected in uterine wet weight, was more pronounced for pigs treated with estrogen during the growth period than as infants or adults (Spencer et al., 1993).

Inappropriate embryonic estrogen exposure results in abnormal oviducts in adult quail and domestic fowl (Greenwood and Blyth, 1938; Rissman et al., 1984; Holm et al., 2001). Abnormalities include retention of the right-side oviduct, reduced size of the left-side oviduct, impaired egg laying, disrupted distribution of carbonic anhydrase in the shell gland, and production of shell-less eggs.

Vitellogenin is potentially an ideal biomarker for measuring the estrogenicity of chemicals (Heppell et al., 1995). The presence of this estrogen-inducible protein in the serum of an animal can be taken as evidence of exposure to endogenous or exogenous estrogens or estrogen mimics (Heppell et al., 1995, Schjeide et al., 1963; Greengard et al., 1964, 1965). Vitellogenin can be measured in blood samples by a number of methods including specific ELISA techniques (Bon et al., 1997).

The deposition of medullary bone, a storage deposit for calcium in the hen, in advance of its being required for eggshell formation, is under estrogenic control

(Dawson, 2000). The formation of medullary bone in immature chickens would indicate an inappropriate exposure to estrogen.

The literature contains very few reports on the estrogenicity of isoflavones in poultry species. Considering that developmentally inappropriate estrogen exposures cause significant abnormalities in mammals and birds, it is important to determine whether naturally occurring estrogenic compounds prevalent in commercial poultry diets alter reproductive development and subsequent reproductive efficiency of domestic poultry. The objective of the study was to examine the estrogenicity of the soy isoflavone genistein in the immature chicken through stimulation of the growth response of the chick oviduct, induction of ovalbumin in the oviduct, the vitellogenin response of the liver, and through deposition of medullary bone in the femur.

Materials and Methods

One hundred one day-old female broiler chicks were obtained from a commercial hatchery. Ten birds were randomly assigned to each of ten battery brooder cages as treatment groups. Birds were housed in an environmentally controlled room and maintained at a room temperature of 31°C (88°F) with continuous light. A small chick waterer was provided for the first five days, after that trough waterers and feeders were used.

Birds were fed a*d libitum* a pelleted and crumbled corn/egg white starter diet (Table 4) with a low level of isoflavones. The diet had a crude protein content of 18.5% and supplied 2870 Kcal/ME/kg. The egg white used in the diet was freeze-dried raw egg white. Since raw egg white contains avidin, which binds biotin, extra vitamin premix was added to the diet to prevent a biotin deficiency. Management and experimental

treatment of the chicks was approved by the Auburn University Instututional Animal Care and Use Committee.

As appropriate for the assigned treatment group, birds were gavaged daily for 14 days with either 1 mg diethylstilbestrol (DES) as a positive estrogen control; 2 mg genistein (G2); 20 mg genistein (G20); 40 mg genistein (G40); or control vehicle (CV) as a negative control. Genistein was purchased from LC Labs, Woburn, MA, USA. All treatments were dissolved in 0.2 ml corn oil vehicle. Daily dosing was carried out using a 1 ml tuberculin syringe and a 16 gauge, 2.5 cm gavage needle. On the 15th day of the trial, one replicate group from each of the five treatments received an injection of 2 mg progesterone dissolved in 0.1 ml corn oil. The progesterone injections were carried out using a 1 ml tuberculin syringe and a 21 gauge, 1 cm needle and were confined to the subcutaneous tissue on the back of the neck.

At 16 days of age, birds in each group were individually weighed, and blood was collected from the ulnar vein into heparinized tubes and stored on ice. Tubes of blood were centrifuged at 1500 RPM for fifteen minutes and the plasma was collected, aliquoted into tubes and stored in a -20°C freezer. All birds were euthanized by CO_2 asphyxiation. The liver, ovary, oviduct, and both femurs were collected from each bird.

Oviducts were weighed and recorded as a percentage of final body weight. Half of the organs that were collected for each treatment group were fixed in 10% neutral buffered formalin and refrigerated for subsequent histological analysis. The other half of the samples were quick-frozen in liquid nitrogen and then transferred to a -80°C freezer. Femurs were placed in sealed plastic bags and stored in a -20°C freezer.

Two of the formalin stored oviduct samples from each treatment were dehydrated in a graded alcohol series and embedded in paraffin, and cut into 3 μm thick sections cut and mounted on slides. Each section contained the entire length of the oviduct. Sections were stained with hematoxylin and eosin for visualization of morphology. Other sections were subjected to immunohistochemical staining for specific proteins. The immunostaining of paraffin sections with microwave antigen retrieval procedure (Appendix 1) was followed.

All tissue sections were stained with commercially available antibodies. The primary antibody in the stain to identify ovalbumin production was monoclonal anti-chicken egg albumin (Sigma, St. Louis, MO) diluted 1:250. The primary antibody for the Proliferating Cell Nuclear Antigen (PCNA) stain was monoclonal anti-recombinant rat PCNA protein (Lab Vision, Freemont, CA) diluted 1:400. The primary antibody for progesterone receptor was monoclonal anti-human endometrial carcinoma progesterone receptor (Lab Vision, Fremont, CA) diluted 1:100. The primary antibody for estrogen receptor alpha was monoclonal anti-human estrogen receptor (Lab Vision, Fremont, CA) diluted 1:100. The secondary antibody for all of the stains was biotintylated goat anti-mouse IG-g (Lab Vision, Fremont, CA). All stains were visualized with streptavidin peroxidase and diaminobenzidine (DAB) (Lab Vision, Fremont, CA).

For analysis, femurs that were collected from all treatments were allowed to thaw overnight in a standard refrigerator. Any excess muscle tissue was removed. A femur from each bird was weighed and the wet femur weight and relative wet weight were recorded. The femurs were then placed in a drying oven for 48 hours at 50°C. Estrogen can have an effect on the wet weight through effects on the amount of bone marrow in

the bone. The difference in weight between the wet femur and dry femur would be an index of the amount of bone marrow in the bone.

Once removed from the drying oven, the femurs were placed in a dessicator and allowed to cool to room temperature. The dry weights of the femurs were recorded and the relative dry femur weights were calculated. The femurs were then placed in crucibles and ashed at 600°C for 18 hours. The femurs were ashed to determine if any of the treatments had an effect on the mineral content, and by implication, the medullary bone in the femur. Medullary bone is the part of the femur that the adult chicken uses to store calcium for eggshell production. The ashed femurs were then placed in a dessicator and allowed to cool to room temperature. The ash femur weights were recorded and the relative ash femur weights were calculated.

A protein analysis was performed on pooled plasma samples to determine amount of protein as a reference for the vitellogenin analysis. The protein analysis was performed following the Bio-Rad Protein Assay protocol (Appendix 2) (Bio-Rad Inc.).

An enzyme linked immunosorbent assay (ELISA) was performed to determine the amount of vitellogenin protein in the plasma samples. The direct ELISA protocol (Appendix 3) was used for this procedure. The primary antibody was monoclonal mouse anti-bird vitellogenin (Biosense Laboratories, Norway) diluted 1:2000. The ELISA was performed with VECTASTAIN Elite ABC Kit (Mouse IgG) and was visualized with ABTS Substrate Kit (Vector Labs, Burlingame, CA). The absorbance was read on a microplate reader at 405 nm and a positive control for this ELISA was plasma from adult hens confirmed to be laying eggs. Plasma vitellogenin concentration was expressed as a percent of control.

Statistical relationships were evaluated using SAS statistical software (SAS Institute, 2002). One-way ANOVA and Tukey's Studentized Range (HSD) Test were conducted to determine any statistical differences. Statistical differences were determined to be significant at a P value of 0.05 or less. All percentage data were subjected to arc sine square root transformation prior to analysis.

Results and Discussion

Typically, broiler chickens grown to 14 days will have consumed approximately 500 g of feed with 35% of that being soybean meal. In commercial practices, defatted soybean meals will contain essentially all of the isoflavones or isoflavone glycosides present in the starting soybeans (Eldridge and Kwolek, 1983). One gram of soybean meal has an approximate average of 150 µg of daidzein and 250 µg of genistein (Dixon and Ferreira, 2002). However, isoflavone content ofsoybeans can vary widely from crop to crop. Assuming an estrogenic equivalency for genistein of about $1/1200^{th}$ the potency of estradiol, derived from values reported in the literature (Reinli and Block, 1996, Korach et al., 1997), this would be equivalent to the average broiler chicken consuming about 0.036 mg of estradiol over 14 days. This level of estrogen exposure has been reported to produce significant effects on reproductive development in mammals (Levy et al., 1995).

The total amount of genistein given to birds during the 14-day trial for the 2 mg, 20 mg, and 40 mg treatment groups was 28 mg, 280 mg, and 560 mg, respectively. These amounts, expressed as estrogen units, were equal to approximately 0.00167 mg, 0.0167 mg, and 0.033 mg of estradiol daily. Over the 14 days of treatment, it would be

equal to 0.0233 mg, 0.233 mg, and 0.467 mg of estradiol, respectively, with these values being within the range of dietary exposure of chickens in commercial production.

There were no significant differences in the starting body weights for any of the treatments (P= 0.1689). This was expected since all of the birds were obtained from the same hatchery at the same time. Birds receiving the genistein treatments had significantly higher (P= 0.0390) final body weights than birds recieving the DES or CV treatments (Table 5). Birds that recieved the DES treatments had significantly higher (P= 0.0390) final body weights than birds recieving the CV treatment (Table 5).

Birds that received the DES treatments had significantly higher oviduct weights (P< 0.0001) and relative oviduct weights (P< 0.0001) as compared to the other treatments (Table 6). This effect of DES on the oviduct was expected due to it being a strong estrogen agonist in the chick. There was no significant effect of genistein on oviduct weight. The doses genistein given in the treatments may have been too weak and/or not enough was absorbed from the gastrointestinal tract.

No statistical differences were found for any of the treatments when comparing wet femur weights (P= 0.2474) or relative wet femur weights (P= 0.5951) (Table 7). Birds that recieved the G20 treatments had signifigantly higher (P= 0.0214) dry femur weights than the CV treatments (Table 8). There were no other significant differences between treatment groups for dry femur weights (P= 0.0214) or relative dry femur weights (P= 0.9086) (Table 8). There were no significant differences between the treatment groups for ashed femur weights (P= 0.0795) or relative ashed femur weights (P= 0.9609) (Table 9).

The CV treatments had significantly higher plasma protein content than all other treatments (P< 0.0001) (Table 10). The G20 treatments had significantly higher plasma

protein content (P< 0.0001) than the DES treatment (Table 10). There were no significant differences (P= 0. 8413) in the vitellogenin content of the plasma as determined by the direct ELISA (Table 10).

DES increased oviduct size as well as the amount of glandular tissue present (Figure 5). This was expected due to the estrogenic activity of genistein. The increase in oviduct size was accompanied by an apparent increase in cellular proliferation as evidenced by PCNA staining. While genistein treatment did not significantly increase oviduct size, it caused an apparent increase in glandularity and cellular proliferation. The DES and G40 treatments had an effect on relative intensity of the PCNA stain (Figure 6). All of the oviduct samples showed some specific staining for PCNA, which was expected since the birds were two weeks of age and growing.

The density of staining for estrogen receptor alpha appeared to be greater with the DES and G40 treatments (Figure 9). High genistein and DES doses presented a higher density of staining for progesterone receptor in the oviduct as compared to the other treatments (Figure 7). This indicates that these treatments induced progesterone redceptor.

The amount of staining visible for ovalbumin production was darker with the DES and G40 treatments (Figure 8). The DES treatments appeared to have secreted ovalbumin visible in the lumen of the oviduct. The CV treatments showed no specific staining for ovalbumin production.

The liver is the site of synthesis for the components of egg yolk. Exposure to estrogenic compounds is known to induce liver synthesis of yolk components such as vitellogenin.

The ovary and oviduct are the main components of the female reproductive tract. Stimulation of growth in these organs by genistein could be determined by weight. The femur is a storage site for calcium in egg-laying birds. Early sexual development may cause calcium to be stored in these bones prior to the onset of lay.

Results of this trial show that genistein has relatively little effect on the reproductive development of the chick when given orally at this age. The only effects of genistein that were visible in this study were found in the staining of slides for specific estrogen inducible proteins. These results could be due to the levels of genistein given to the birds during this trial. Genistein has been shown to have weak estrogenic activity as compared to both estradiol and diethylstilbestrol. The levels of genistein chosen for this experiment may have been too low to show a strong estrogenic effect on the chick development.

The effects from the treatments with genistein may not have had enough time to appear. The trial started with day-old chicks and ran for only two weeks. This short period of dosing may not have been enough to alter reproductive development. It is possible that a longer trial or older birds would have had a different outcome.

It has been shown that genistein and other isoflavones are readily metabolized in the gut of animals. If genistein is not absorbed into the body, it is further metabolized into p-ethylphenol (Barrett, 1996). This is a hormonally inert compound. It is possible that the oral dosing of genistein showed little effect on the reproductive development of the female chick due to the metabolism of the animal. The genistein that was given in the treatment may have been changed into the inert compound or simply excreted from the animal before it could have a chance to cause any developmental changes.

Orally ingested genistein appears to have some estrogenic potency in the chick, as compared to a known, highly potent synthetic estrogen. In the chick oviduct model system, genistein induced the appearance of estrogen and progesterone receptors, increased cellular proliferation, and promoted the ability of progesterone to induce ovalbumin synthesis. However, at the doses used in this study, genistein did not induce significant growth of the oviduct as did DES, nor did it significantly increase plasma vitellogenin, which is a common marker for estrogenic exposure in a number of species. Thus, it appears that ingested genistein is only weakly estrogenic in the chick.

The relatively weak effects of genistein as compared to DES could be due to a number of factors. Genistein is poorly absorbed from the intestine of most animals, is subject to bacterial conversion, and is rapidly metabolized in the hepatic circulation and conjugated to bile for excretion. Furthermore, it is possible that genistein does not possess the full range of activity of steroidal estrogens. Many estrogenic substances exhibit both estrogen agonist and antagonist activities through competition for receptors and plasma binding proteins. This may have been the case with genistein in this study. It is also possible that more pronounced estrogenic effects would have been induced with a longer duration of dosing. It is possible that the chicken reproductive system may be more responsive to genistein during a different period of development.

TABLE 4. Ingredient percentages and calculated analysis of broiler starter diet with a low level of isoflavones.

Ingredient	(%)
Corn	78.65
Egg Albumin (82% CP)	13.23
Soybean Meal (48% CP)	3.00
Rice Mill Feed	1.38
Dicalcium Phosphate[1]	1.37
Limestone (38% Ca)	1.35
Vitamin Premix[2]	0.75
L-Lysine	0.25

Trace Mineral Premix[3]	0.25
Salt	0.02
Calculated Analysis	
Crude Protein (%)	18.15
Fat	3.101
Fiber	2.542
ME (kcal/kg)	2870.00
Calcium (%)	0.95
Available Phosphorus (%)	0.45
Methionine (%)	0.60
Methionine & Cystein (%)	1.00
Lysine (%)	1.15
Arginine (%)	1.14
Threonine (%)	0.82

[1] Contains 18.5% phosphorous and 24.1% calcium.

[2] Supplied the following per kg of complete feed: vitamin A, 24,000 IU (retinyl palmitate); cholecaliciferol, 6,000 IU; vitamin E, 24 IU (di-tocopheryl acetate); menadione, 6 mg; riboflavin, 16.5 mg; pantothenic acid, 39 mg; niacin, 108 mg; choline, 1500 mg; vitamin B_{12}, 0.06 mg; folic acid, 15 mg; thiamin, 3 mg; pyridoxine, 6.6 mg; biotin, 0.15 mg: ethoxyquin, 375 mg.

[3] Supplied the following per kg of complete feed: manganese, 125 mg; iodine, 1 mg; iron, 55 mg; copper, 6 mg; zinc, 55 mg; selenium, 0.3 mg.

TABLE 5. Starting and final body weight.

Treatment	Starting BW (g)	P value	N	Final BW (g)	P value	N
CV	38.9 ± 3.5 [NS]	0.1689	20	218.9 ± 27.1 [c]	0.0390	16
G2	38.1 ± 3.1 [NS]		20	243.5 ± 34.0 [a]		15
G20	37.8 ± 2.6 [NS]		20	247.0 ± 26.7 [a]		17
G40	39.7 ± 2.7 [NS]		20	247.0 ± 22.6 [a]		18
DES	39.0 ± 2.8 [NS]		20	231.7 ± 39.2 [b]		18

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[2] Values shown are treatment averages with standard deviation.

TABLE 6. Weight and relative weight of the oviduct at 16 days of age.

Treatment	Oviduct Weight (g)	P value	N	Relative Weight (%)	P value
CV	0.036 ± 0.01 [b]	< 0.0001	16	0.02 ± 0.02 [b]	< 0.0001
G2	0.040 ± 0.01 [b]		15	0.02 ± 0.00 [b]	
G20	0.038 ± 0.01 [b]		17	0.02 ± 0.00 [b]	
G40	0.041 ± 0.01 [b]		18	0.02 ± 0.00 [b]	
DES	0.329 ± 0.13 [a]		18	0.14 ± 0.05 [a]	

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from Final BW in Table 5.

TABLE 7. Weight and relative weight of one femur from each bird in each treatment at 16 days of age.

Treatment	Wet Femur Weight (g)	P value	N	Relative Weight (%)	P value
CV	1.480 ± 0.267 [NS]	0.2474	16	0.6337 ± 0.069 [NS]	0.5951
G2	1.504 ± 0.236 [NS]		15	0.6094 ± 0.046 [NS]	
G20	1.474 ± 0.246 [NS]		17	0.5732 ± 0.051 [NS]	
G40	1.427 ± 0.128 [NS]		18	0.5785 ± 0.034 [NS]	
DES	1.372 ± 0.160 [NS]		18	0.5951 ± 0.049 [NS]	

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from Final BW in Table 5.

TABLE 8. Weight and relative weight of one femur from each bird in each treatment at 16 days of age after 48 hours at 50°C.

Treatment	Dry Femur Weight (g)	P value	N	Relative Weight (%)	P value
CV	0.563 ± 0.096^{b}	0.0214	16	0.241 ± 0.025^{NS}	0.9086
G2	0.575 ± 0.084^{ab}		15	0.233 ± 0.018^{NS}	
G20	0.586 ± 0.088^{a}		17	0.228 ± 0.017^{NS}	
G40	0.575 ± 0.049^{ab}		18	0.233 ± 0.011^{NS}	
DES	0.537 ± 0.059^{ab}		18	0.233 ± 0.023^{NS}	

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from Final BW in Table 5.

TABLE 9. Weight and relative weight of one femur from each bird in each treatment at 16 days of age after 18 hours at 600°C.

Treatment	Ashed Femur Weight (g)	P value	N	Relative Weight (%)	P value
CV	0.203 ± 0.033^{NS}	0.0795	16	0.087 ± 0.009^{NS}	0.9609
G2	0.212 ± 0.031^{NS}		15	0.086 ± 0.008^{NS}	
G20	0.216 ± 0.033^{NS}		17	0.084 ± 0.006^{NS}	
G40	0.209 ± 0.025^{NS}		18	0.085 ± 0.006^{NS}	
DES	0.199 ± 0.025^{NS}		18	0.086 ± 0.011^{NS}	

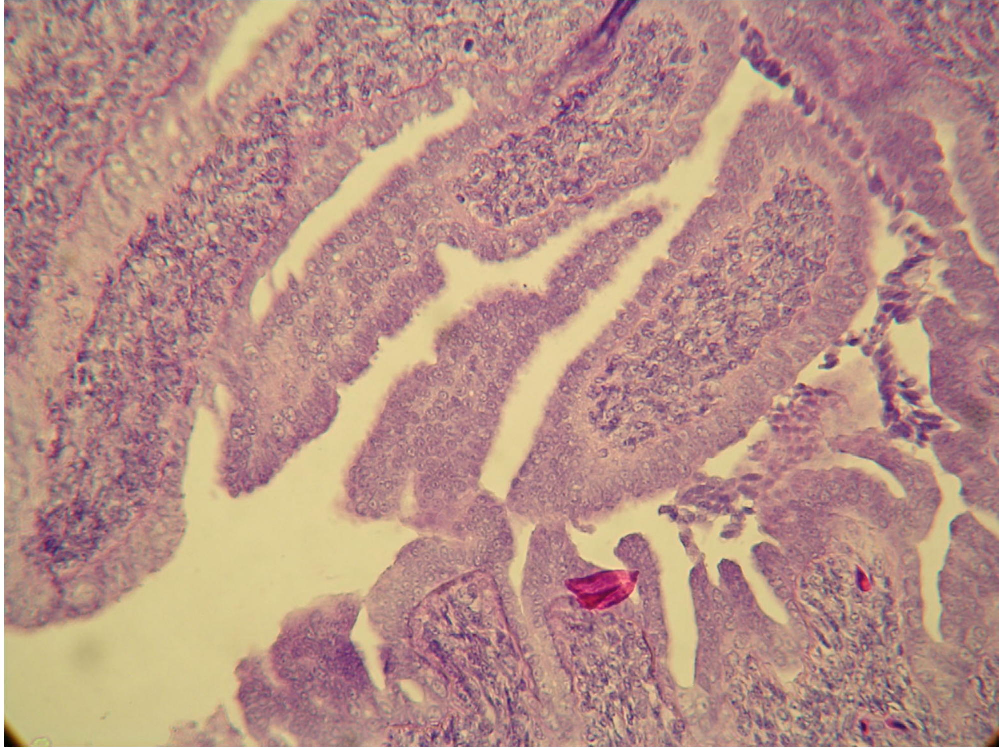
[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from Final BW in Table 5.

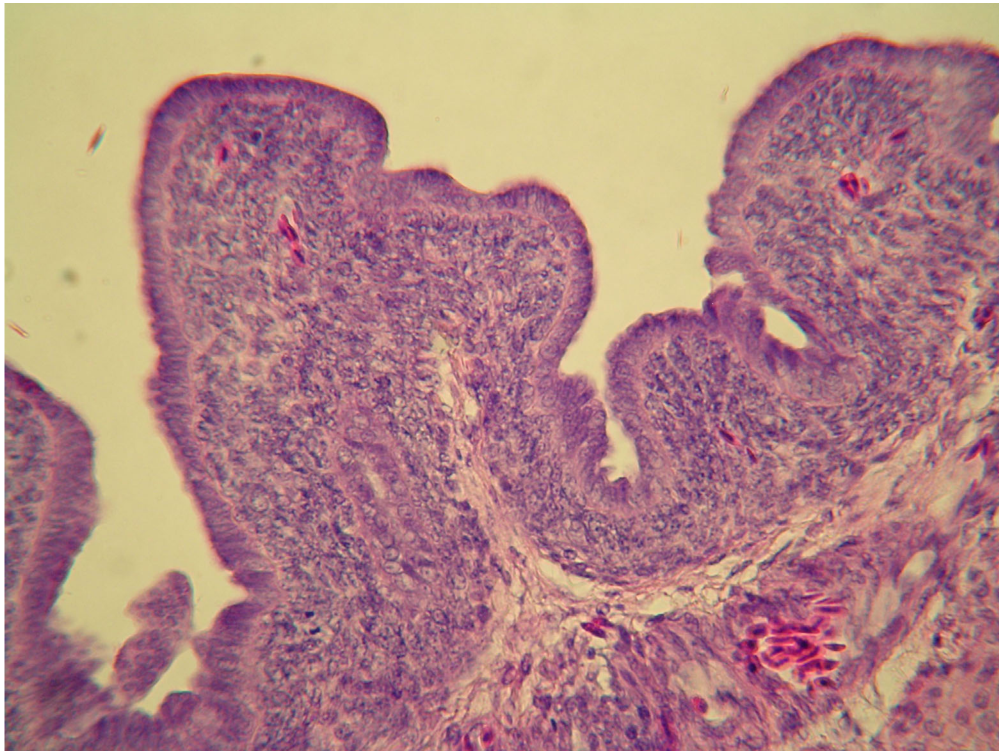
TABLE 10. Protein and vitellogenin concentration in the plasma of birds at 16 days of age.

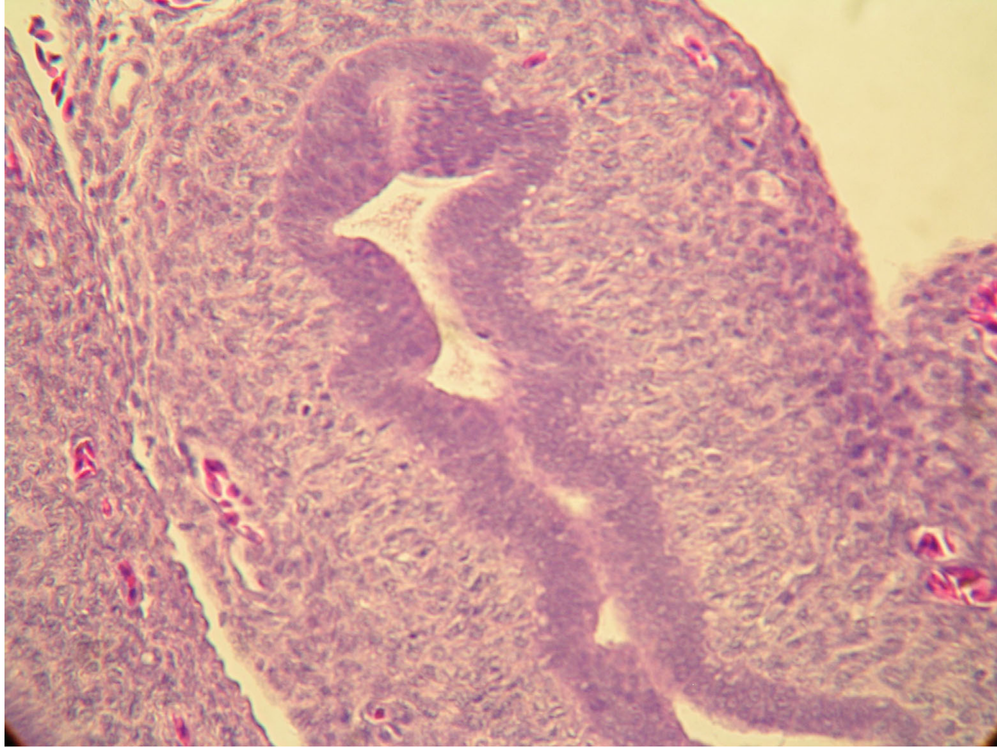
Treatment	Protein (mg/ml)	P value	N	Vitellogenin [3]	P value	N
CV	25.617 ± 4.070^{a}	< 0.0001	4	100.000 ± 1.137^{NS}	0.8413	12
G2	15.109 ± 0.816^{bc}		4	131.171 ± 1.437^{NS}		13
G20	13.779 ± 0.880^{b}		4	109.126 ± 1.435^{NS}		17
G40	15.865 ± 1.098^{bc}		5	124.519 ± 0.947^{NS}		17
DES	18.030 ± 1.708^{c}		6	147.828 ± 1.880^{NS}		10

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from Final BW in Table 5.
[3] Expressed as a percent of the control (CV) and calculated to represent the total protein.

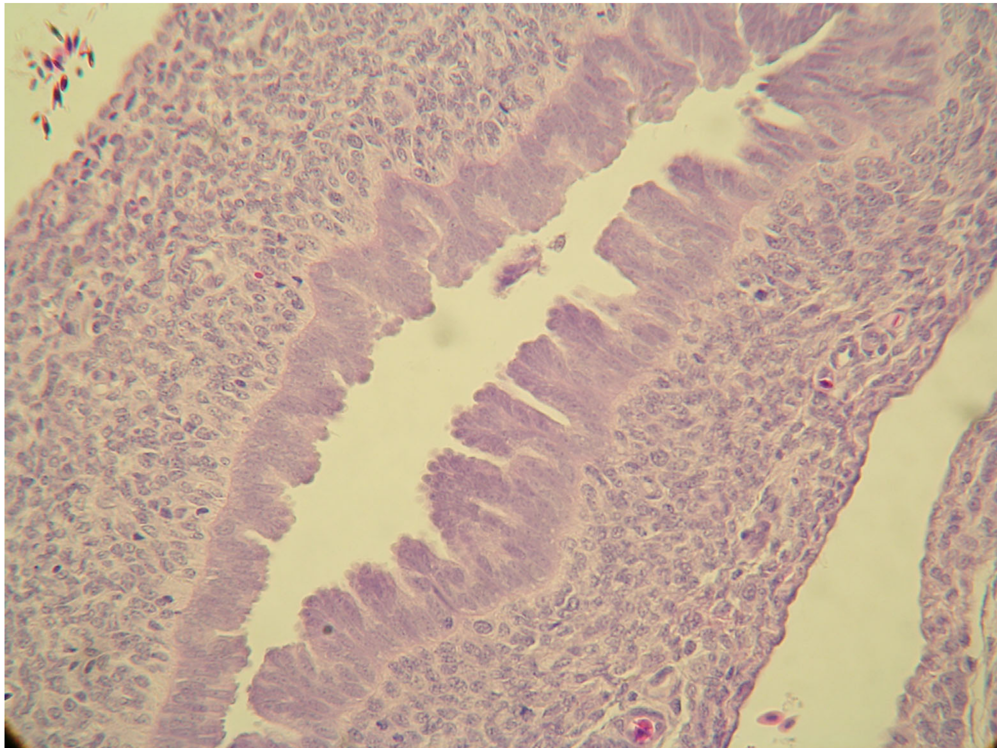
FIGURE 5. Hemotoxylin and eosine stain on paraffin embedded oviduct sections removed at 16 days of age.


CV

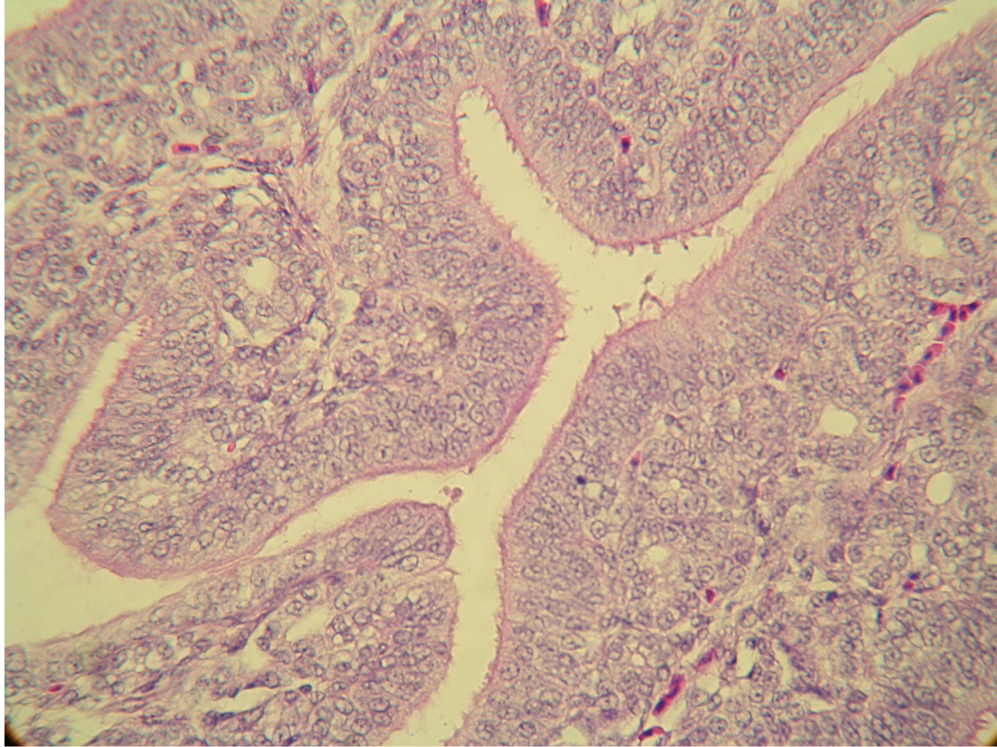

G2



G20

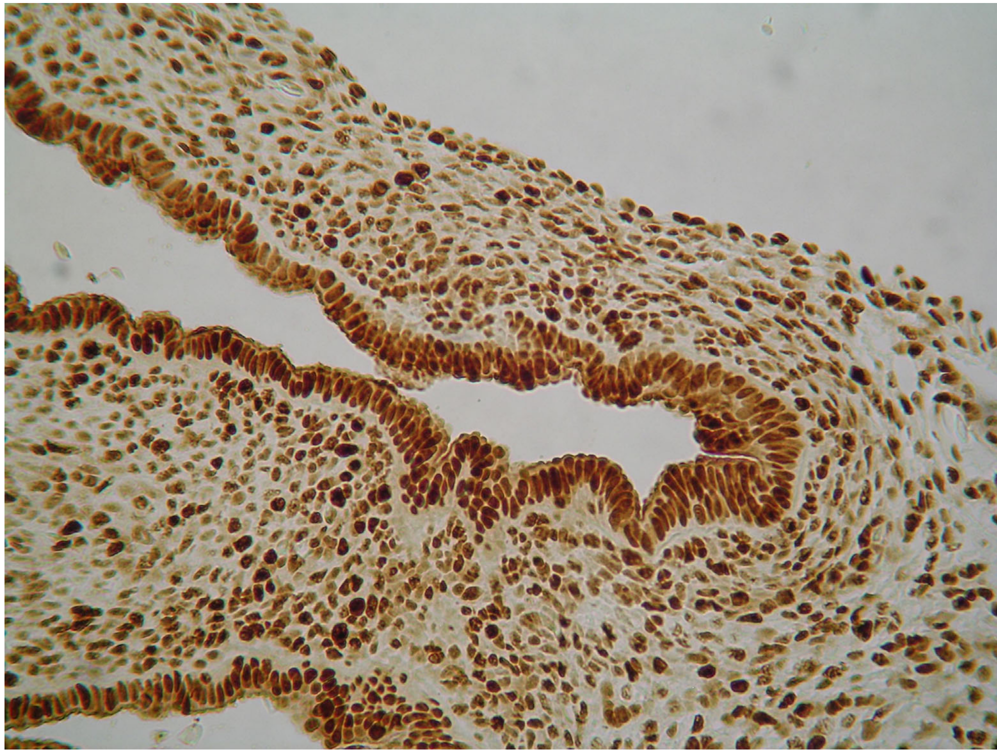


G40

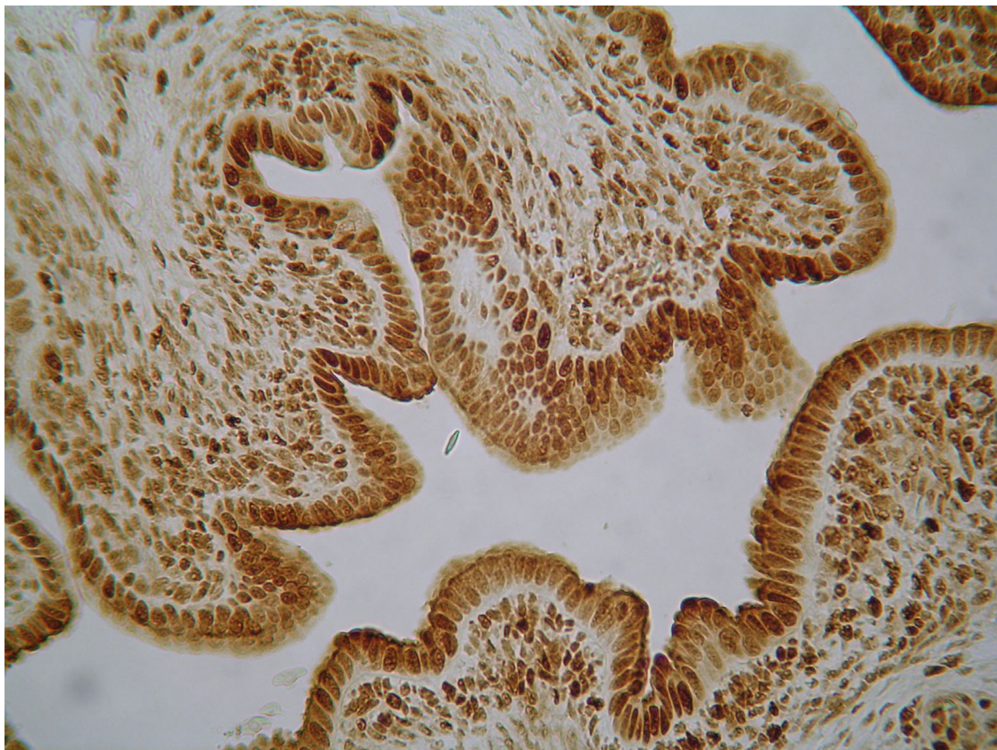


DES

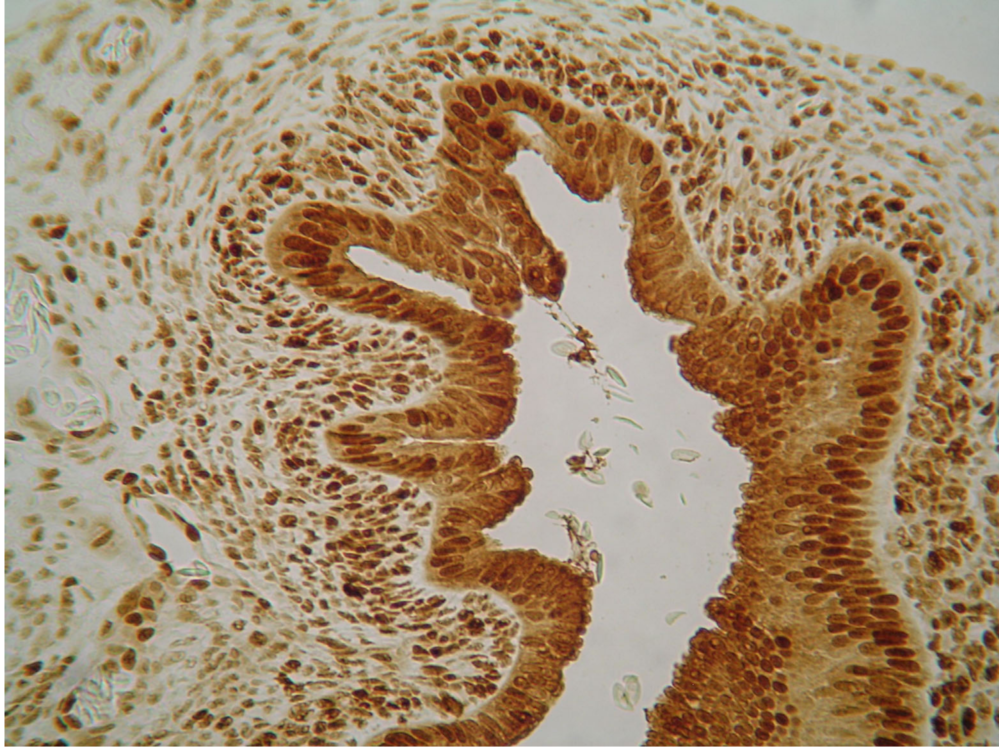
FIGURE 6. Proliferating cell nuclear antigen (PCNA) stain on paraffin embedded oviduct sections removed at 16 days of age.



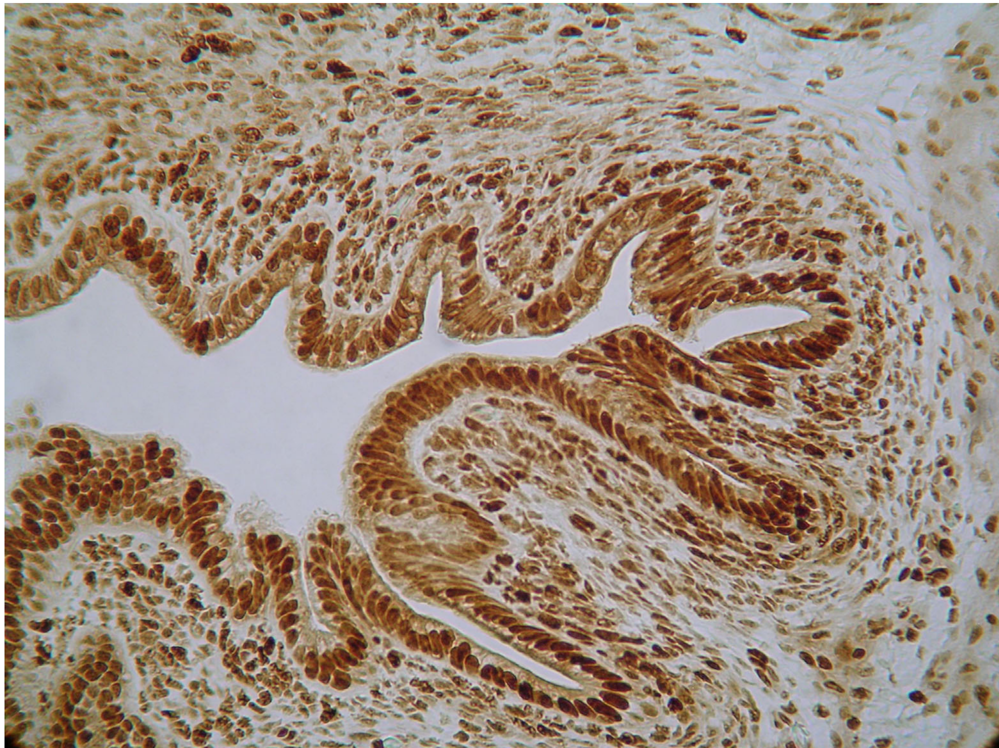
CV



G2



G20



G40

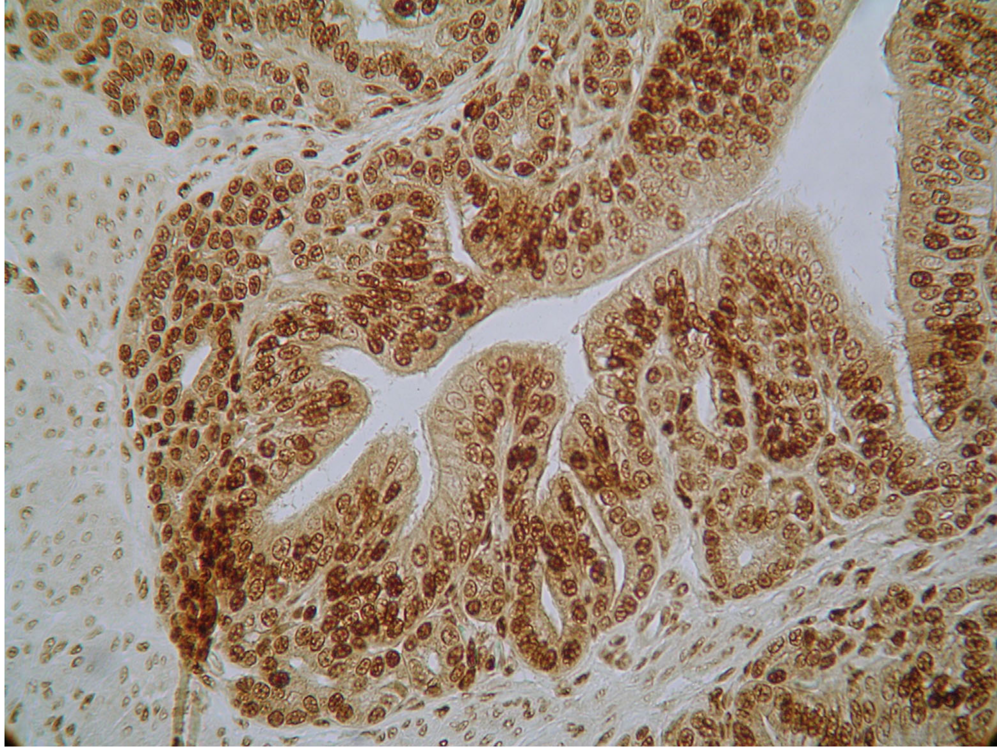
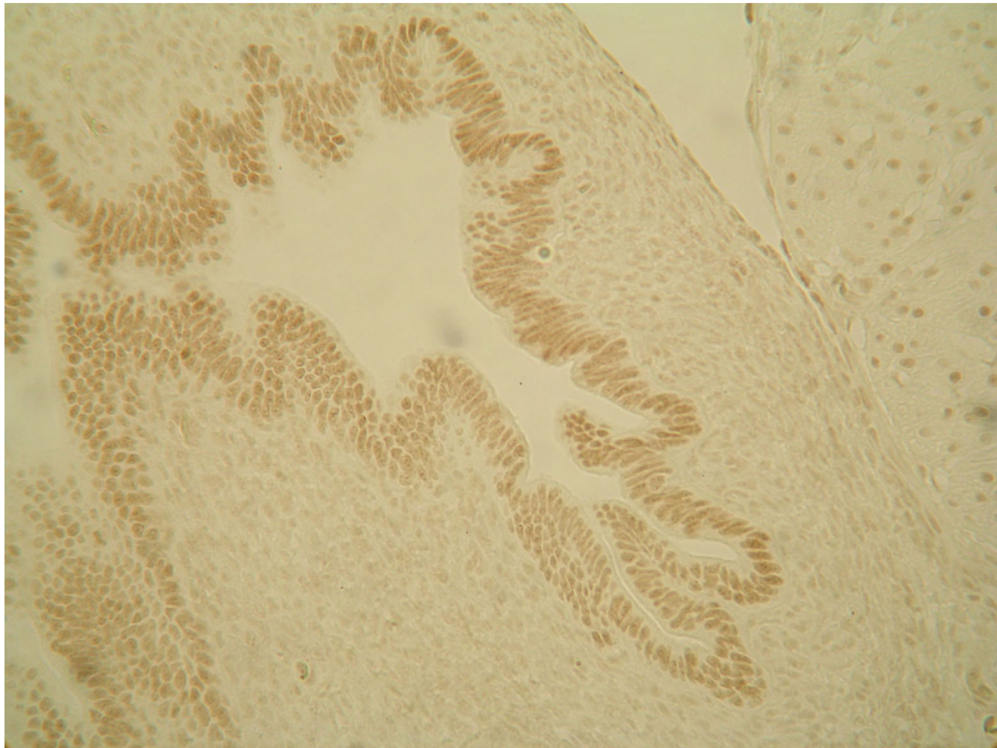
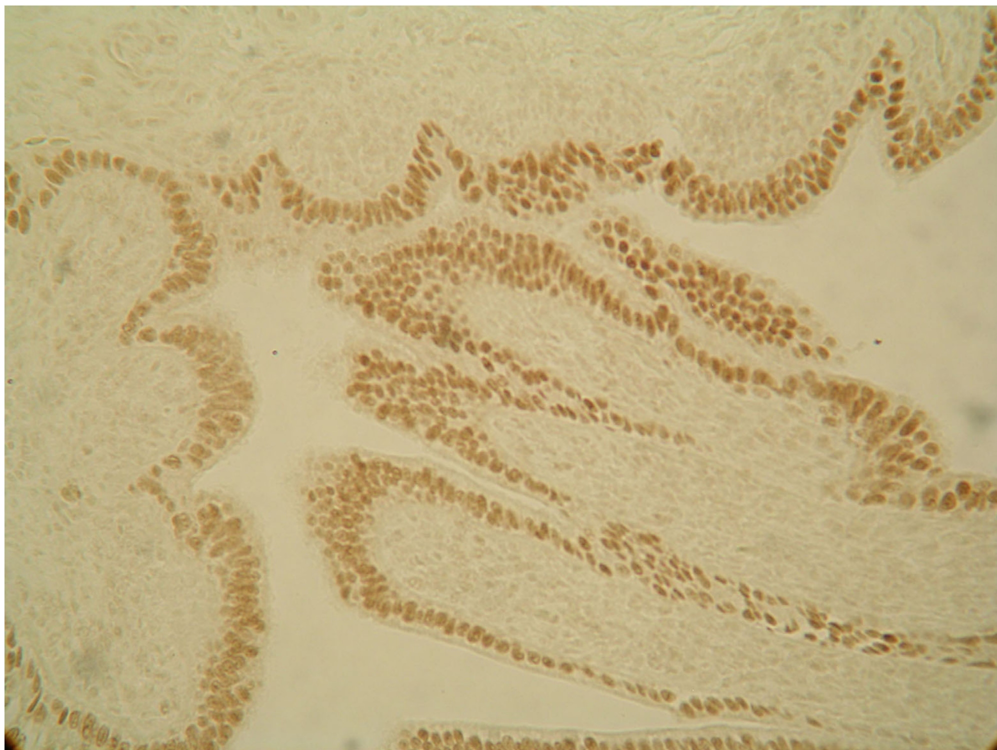

DES

FIGURE 7. Progesterone receptor stain on paraffin embedded oviduct sections removed at 16 days of age.



CV



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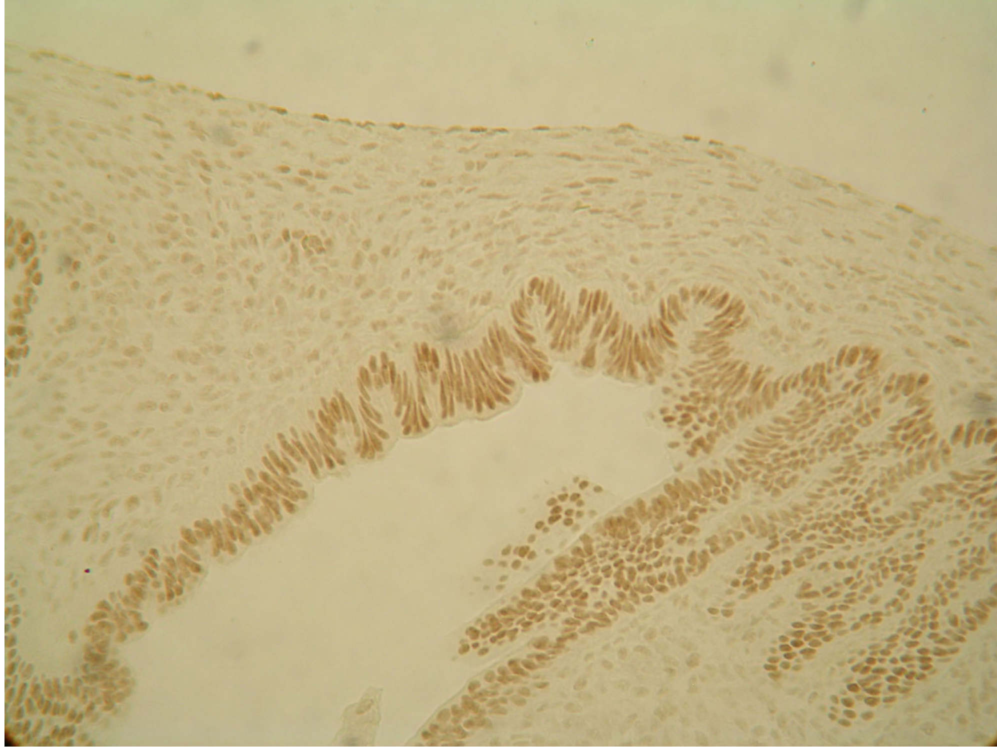
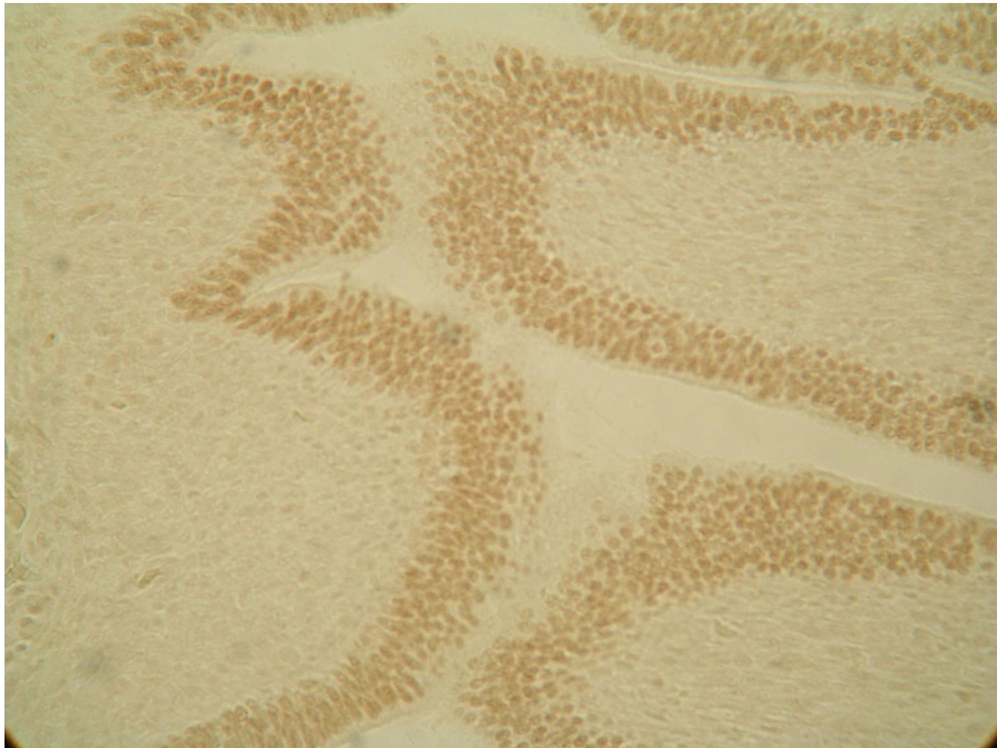

G20


G40

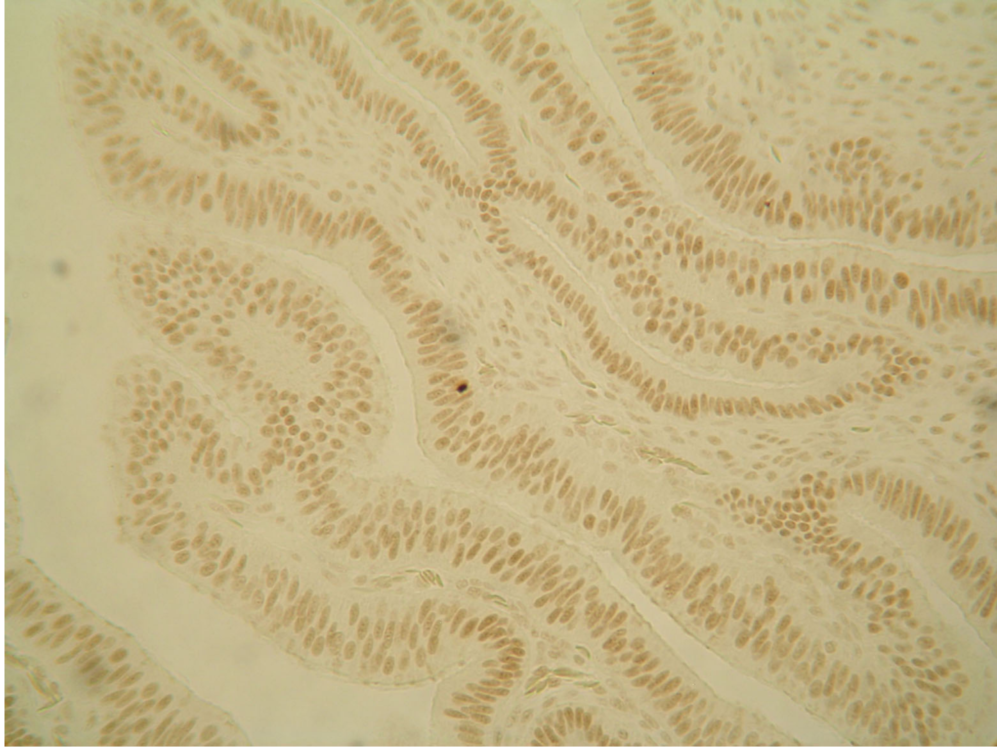
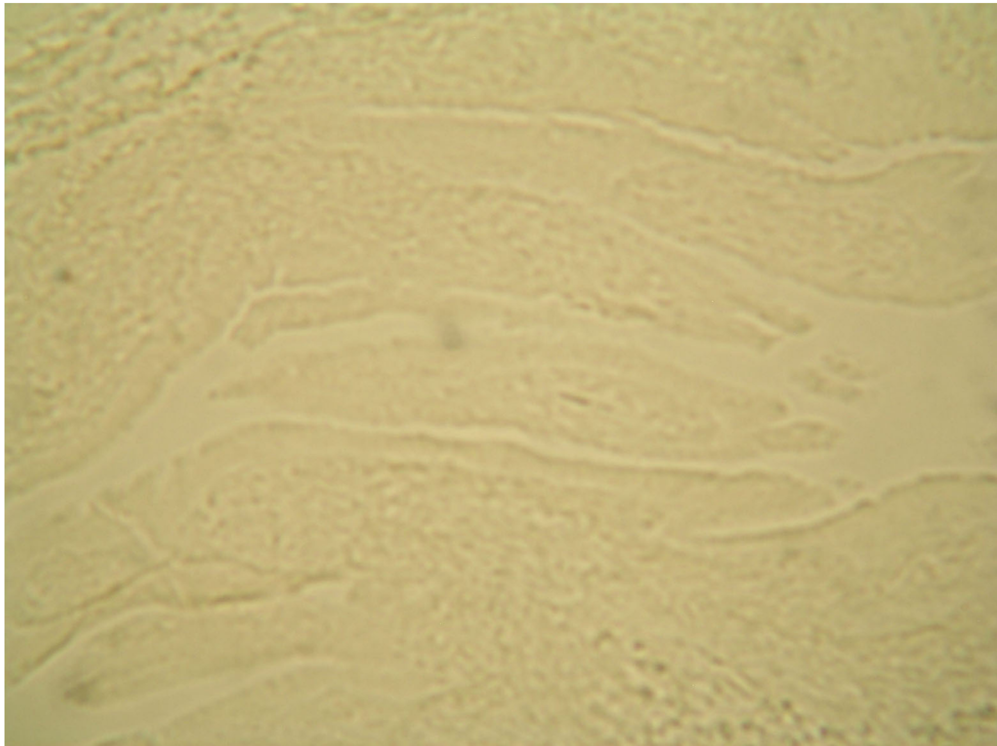
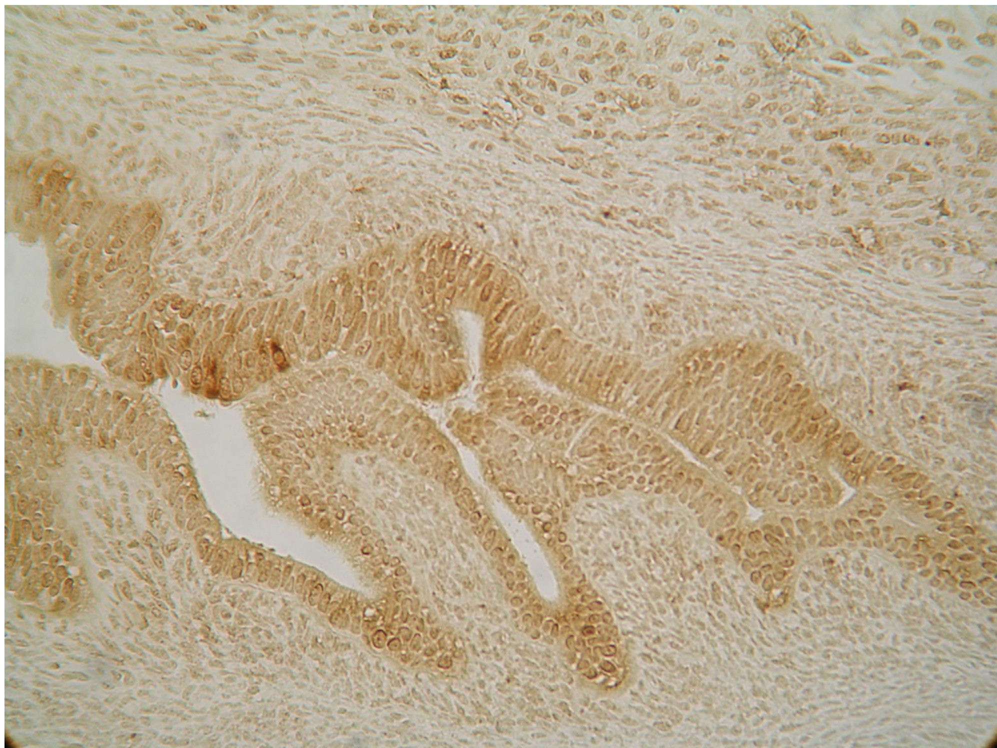

DES

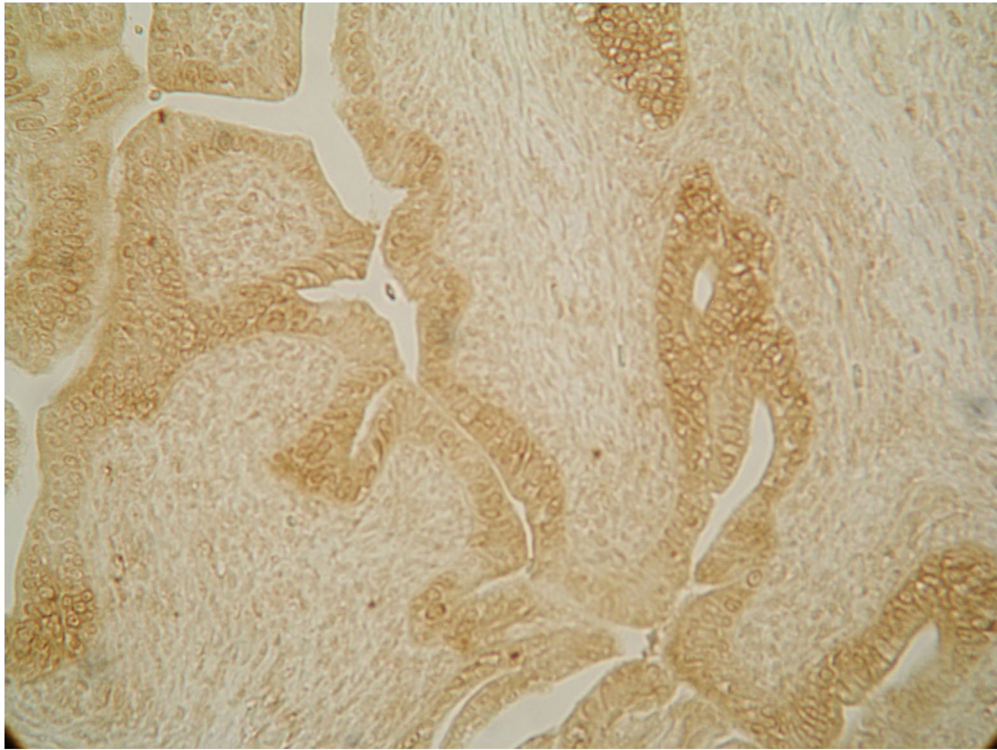
FIGURE 8. Ovalbumin production stain on paraffin embedded oviduct sections removed at 16 days of age.



CV



G2



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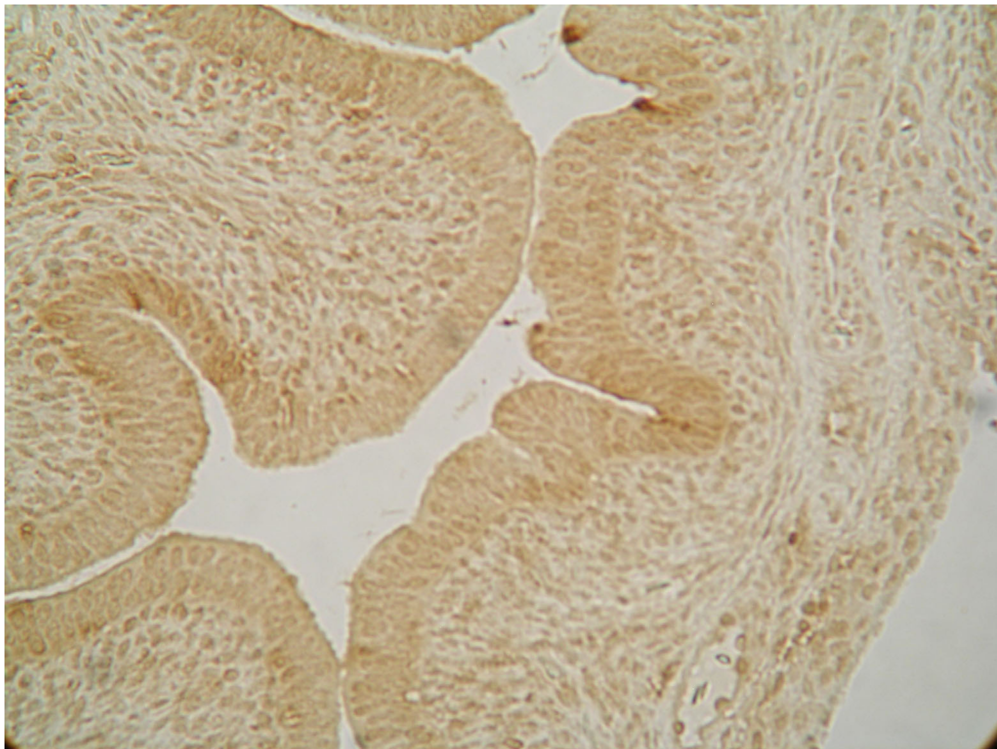
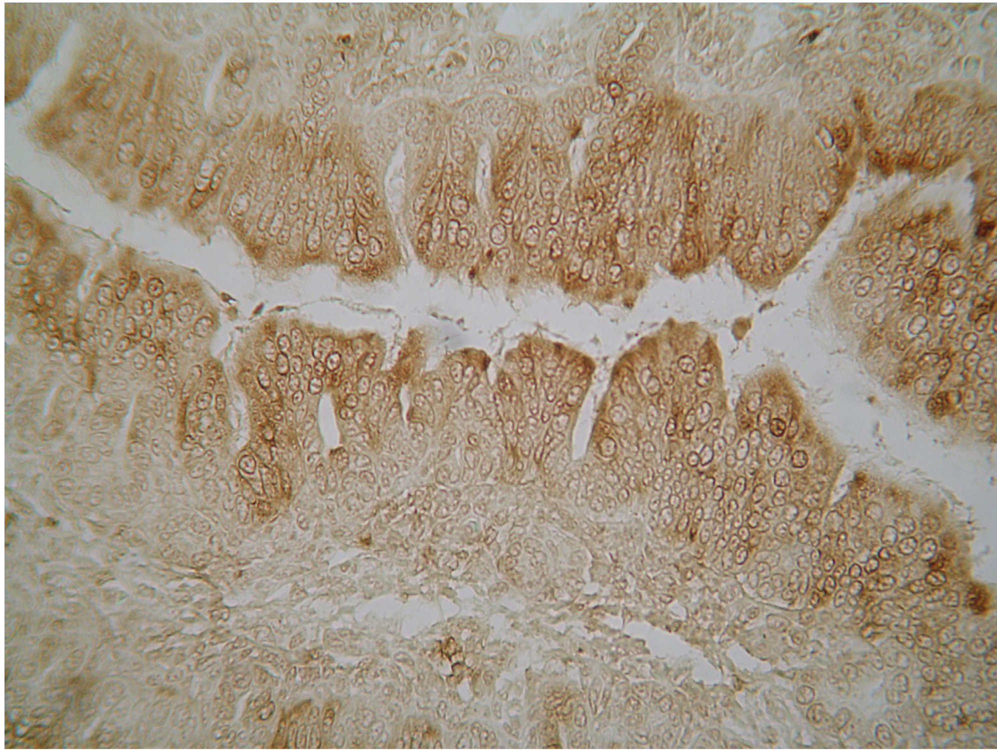
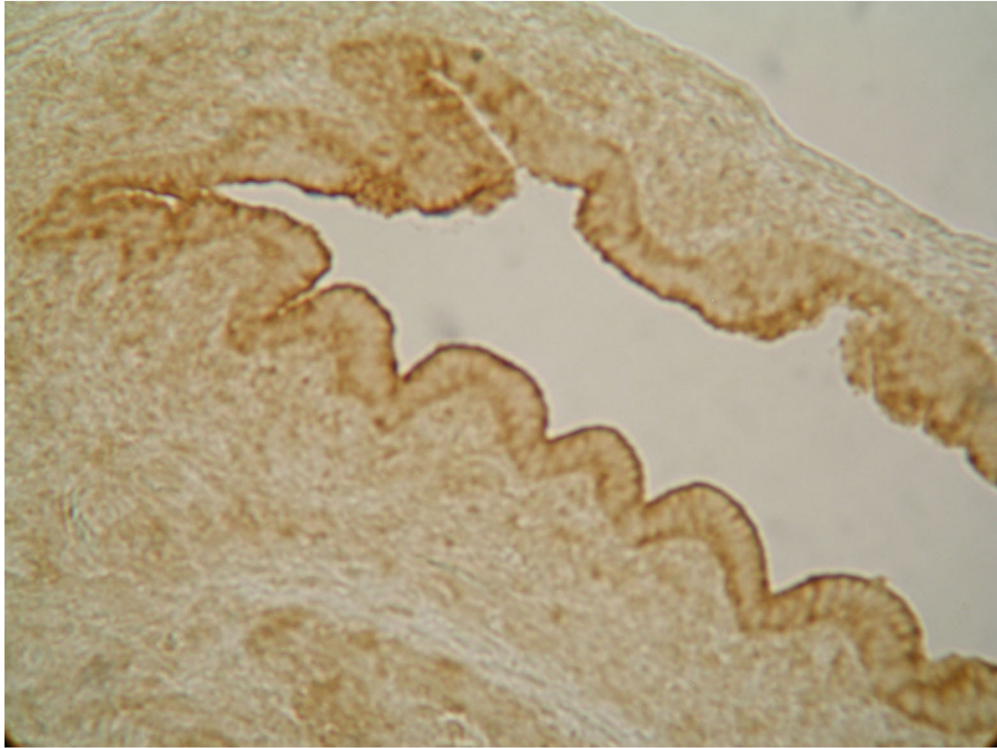


G40

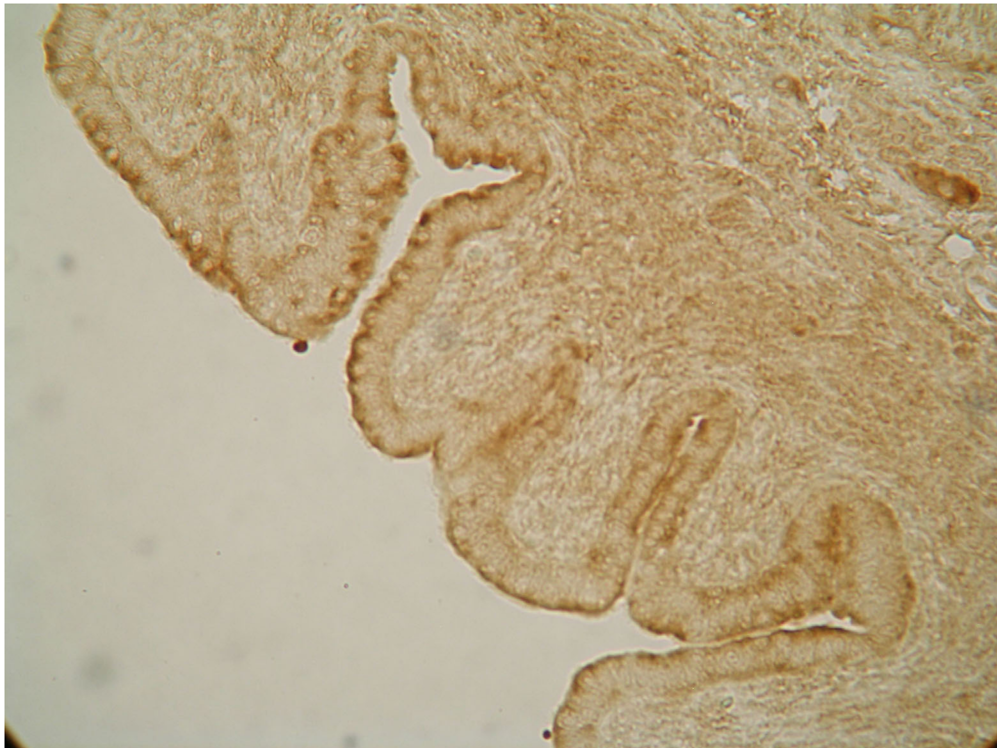


DES

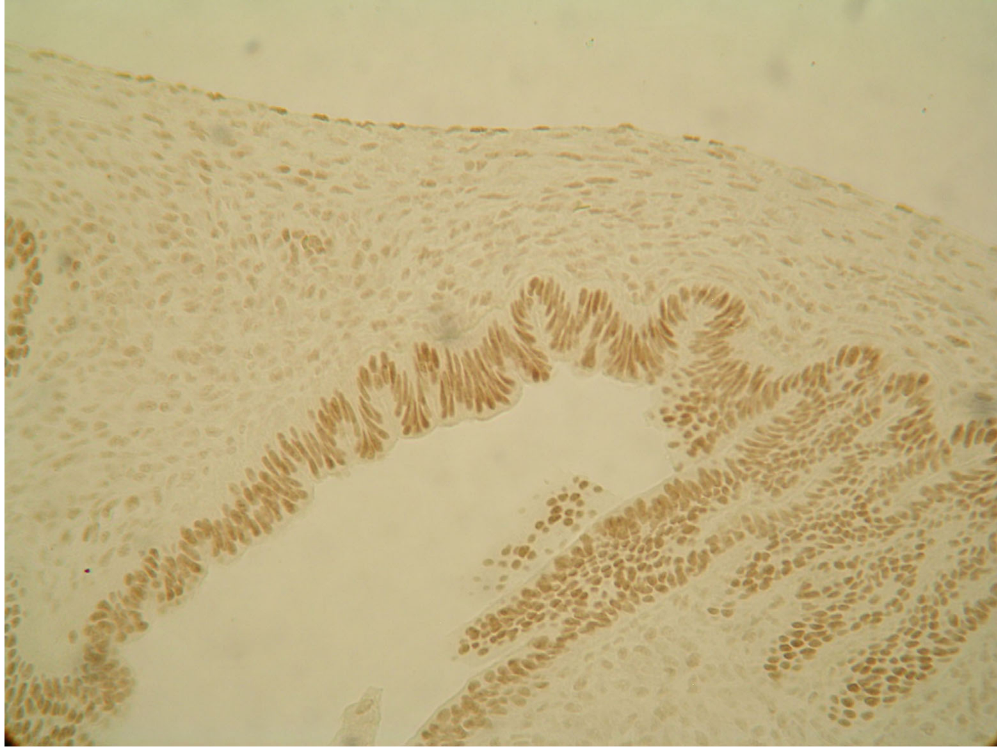
FIGURE 9. Estrogen receptor alpha stain on paraffin embedded oviduct sections removed at 16 days of age.

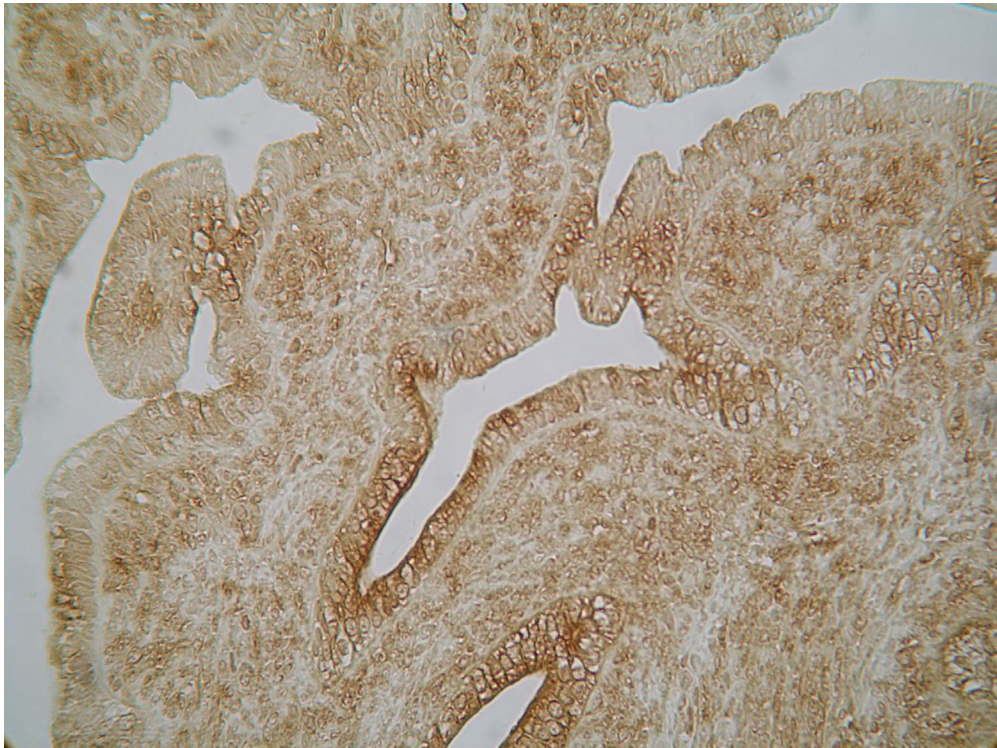


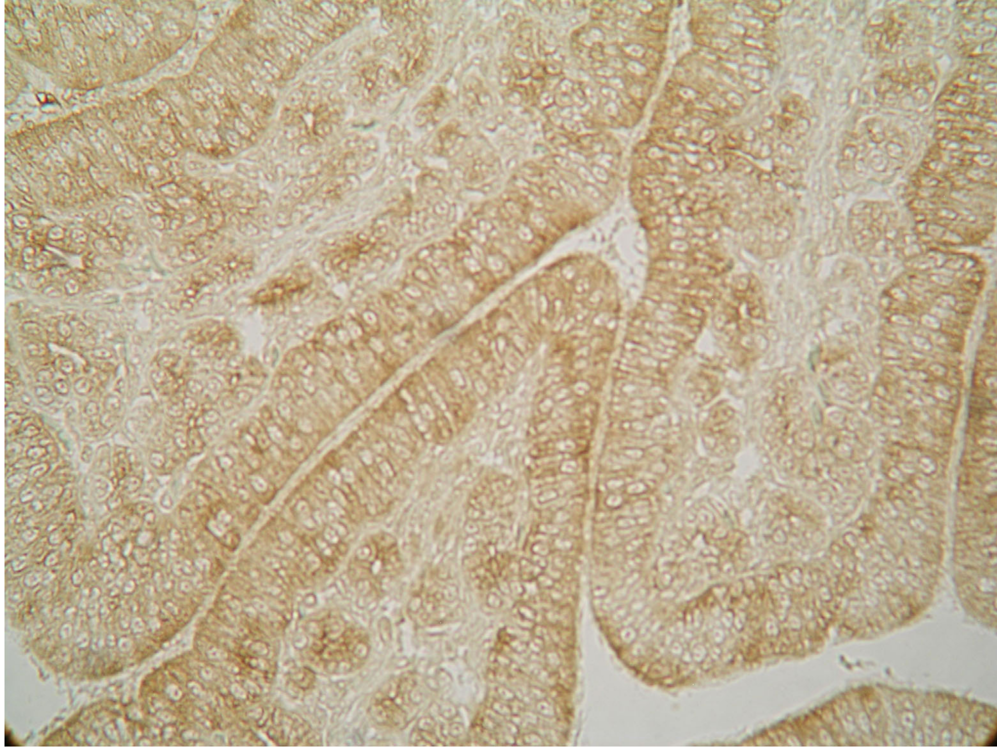
CV



G2


G20


G40


DES

V. MANUSCRIPT 2

EFFECTS OF SHORT-TERM ORAL EXPOSURE TO THE ISOFLAVONE GENISTEIN ON IMMATURE FEMALE BROILER CHICKENS

Abstract

To determine the effects of short-term oral exposure to the estrogenic soy isoflavone genistein on immature female chickens; 100 female chicks were wing banded, individually weighed, and placed into battery brooders. Chicks were assigned to ten treatment groups. Two replicate groups per treatment received a daily gavage of either 0.2 ml sesame oil vehicle (SV) (CV); 1 mg diethylstilbestrol (DES); 10 mg genistein (G10); or 40 mg genistein (G40). The CV, G10, G40, and DES groups were fed an egg-white based chick starter diet. The SV group was fed a standard chick starter diet. At 15 days of age, DES treatment increased oviduct weight and relative oviduct weight as compared to all other treatments ($P<0.05$). The standard diet increased body weight and weight gain, and decreased relative liver weight ($P<0.05$) as compared to the other treatments. These results indicate that genistein acts as a weak estrogen with selective effects on the immature chicken oviduct.

Introduction

Estrogens are endogenous steroid hormones with numerous physiological actions (Norris, 1985; van Tienhoven, 1983; Korach et al., 1997; Bennink and Boerriter, 2003). They are formed by the aromatization of androgens by steroidogenic enzymes. The structure of steroid hormones is very important. A phenolic ring structure is necessary for estrogen-like activity (Korach et al., 1997). Estrogen influences the growth, development, behavior and regulation of reproductive tissues in all vertebrates (Matthews et al., 2000; Zava and Duwe, 1997; Peterson and Barnes, 1996; Femo et al., 2000).

Environmental estrogens are estrogens that are not normally present in an animal. Many of these are naturally occurring compounds synthesized by plants, natural steroidal estrogens or synthetic estrogens used in medicine that were excreted into the environment, or chemicals that were synthesized for another purpose and then found to have estrogenic activity (Burton and Wells, 2002). Environmental estrogens have been implicated in the pathogenesis of hormonally treated cancers, male infertility, and abnormalities of both the male and female reproductive tracts (Burton and Wells, 2002).

Endocrine modulators that are produced synthetically have been given the term 'endocrine disrupting chemicals' (EDC) (Matthiesen et al., 1996; Gutendorf and Westendorf, 2001). Estrogenic compounds created by plants are referred to as phytoestrogens. These are plant chemicals that resemble natural estrogens in either their structure or function (Whitten and Naftolin, 1998; Bradbury and White, 1954). Phytoestrogens have the ability to mimic som, or all of the actions of endogenous estrogens. They can act as estrogen agonists or antagonists. They can also alter the pattern of synthesis and/or metabolism for the endogenous hormones (Sonnenschein and Soto, 1998; Whitten et al., 1995).

The isoflavonoid class, which contains isoflavones and isoflavans, is the largest class of phytoestrogens (Korach et al., 1997). The four most common isoflavones are formononetin, daidzein, genistein, and biochanin A (Barrett, 1996; Thomas, 1997). Genistein is biosynthetically the simplest of the isoflavonoid compounds of the leguminosae (Dixon and Fierriera, 2002).

Isoflavones are each found in four chemical forms. The unconjugated forms or aglycones include daidzein, genistein, and glycitein (Kudou et al., 1991). The glucosides are plant sugar derivatives (Barrett, 1996). They include daidzin, genistin, and glycitin. There are also acetylglucosides and malonylglucosides (Kudou et al., 1991). The aglycones may be more bioavailable than glucosides (Kurzer, 1997). The O-methylation of isoflavones decreases their estrogenicity in *in vitro* ER binding assays. Because of this, formononetin and biochanin A are less potent than daidzein and genistein (Dixon, 2004).

Glucosides make up to 50-98% of the isoflavones found in soy foods (Song et al., 1998). In commercial practices, defatted soybean meals will contain essentially all of the isoflavones of isoflavone glucosides that are present in the starting soybeans (Eldridge, and Kwolek 1983). Genistein is naturally deglycosylated and absorbed into the blood stream as 7-O-β-glucuronide, not as genistein (Bennetau-Pelissero et al., 2001; Sfakianos et al., 1997; Yuan et al., 2003). It has been shown that the blood plasma of hens fed an isoflavone-enriched diet contains mostly the conjugated forms of isoflavones and less of the glycoside and aglycone forms (Saitoh et al., 2004). This suggests that the dietary soybean isoflavone-glycosides are deglycosylated in the hen's intestinal tract and absorbed as aglycones. These are then metabolized and conjugated in intestinal epithelial cells and released in the bloodstream as conjugated forms (Saitoh et al., 2004).

Genistein is a central intermediate in the biosynthesis of more complex isoflavonoids (Dixon and Ferreira, 2002). It is a diphenolic planar molecule with an aromatic A-ring. It has a second oxygen atom that is 11.5 Å from the one in the A-ring and a molecular weight that is similar to those of steroidal estrogens (Lamartiniere et al., 1995). It shares structural features with the potent estrogen 17-β estradiol. It is particularly similar in the phenolic ring and the distance between its 4'-and 7'- hydroxyl groups (Dixon et al., 2002). These features allow genistein to bind to the estrogen receptors in animals (Hopert et al., 1998; Milligan et al., 1998; Messina et al., 1994; Makarevich et al., 1997).

Genistein can function as both an estrogen agonist and antagonist in animals (Adlercreutz, 1990). It has been shown that daily administration of estrogen results in a marked increase in the wet weight of the oviduct (Oka and Schimke, 1969).

Quantatative analysis of soy isoflavones in biological fluids can be accomplished with a variety of methods and analytical instrumentation. High-performance liquid chromatography (HPLC) with ultraviolet detection is one of the more common methods. Recently an HPLC-UV method for the quantification of genistein, daidzein, glycitein, and their primary metabolites in human plasma and urine was created. This method was validated with the US FDA guidelines for the validation of methods used in pharmokinetic studies (Thomas et al., 2001).

The literature contains very few reports on the estrogenicity of isoflavones in poultry species. Considering that developmentally inappropriate estrogen exposures cause significant abnormalities in mammals and birds, it is important to determine whether naturally occurring estrogenic compounds prevalent in commercial poultry diets alter reproductive development and subsequent reproductive efficiency of domestic

poultry. The objective of the study was to examine the estrogenicity of the soy isoflavone genistein in the immature chicken by observing the potential stimulation of the growth response of the chick oviduct, amount of genistein, vitellogenin, and protein present in the plasma, and through deposition of medullary bone in the femur.

Materials and Methods

One hundred one day-old female broiler chicks were obtained from a commercial hatchery. Ten birds were randomly assigned to each of ten battery brooder cages as treatment groups. The birds were housed in an environmentally controlled room and maintained at a room temperature of 31°C (88°F) with continuous light. A small chick waterer was provided for the first five days, after that trough waterers and feeders were used. Management and experimental treatment of the chicks was approved by the Auburn University Instututional Animal Care and Use Committee.

Birds in the control vehicle (CV), genistein, and diethylstilbestrol (DES) treatments were fed a pelleted and crumbled corn/egg white starter diet *ad libitum* (Table 11) with a low level of isoflavones. The diet had a crude protein content of 18.5% and supplied 2870 KcalME/Kg. The egg white used in the diet was freeze-dried raw egg white. Since raw egg white contains avidin, which binds biotin, extra vitamin premix was added to the diet to prevent a biotin deficiency. Birds in the SV treatment were fed a standard chick starter diet *ad libitum* (Table 12). The diet had a crude protein content of 23% and supplied 3120 KcalME/Kg.

As appropriate for the assigned treatment goups, birds were gavaged daily for 14 days with either 1 mg diethylstilbestrol (DES) as a positive estrogen control; 10 mg genistein (G10); 40 mg genistein (G40); control vehicle (CV) as a negative control; or

standard vehicle (SV) as a comparison to commercially produced chickens. All treatments were dissolved in 0.2 ml sesame oil rather than corm oil to avoid problems with possible estrogenic mycotoxin contamination of the corn oil. Daily dosing was carried out using a 1 ml tuberculin syringe fitted with a 16 gauge, 2.5 cm gavage needle.

On day 0, 5, 10, and 15, body weights of all birds were determined and the gain per bird was calculated for days 0 to 5, 5 to 10, and 10 to 15. On day 15, blood was collected from the ulnar vein into heparinized tubes and stored on ice. Tubes of blood were then centrifuged at 1500 RPM for fifteen minutes. Plasma was collected, aliquoted into tubes and stored in a 20°C freezer.

Birds were euthanized by CO_2 asphyxiation and necropsied on the day 15. Liver, oviduct, and both femurs were collected from each bird and were weighed. Relative weight was calculated as a percent of final body weight. Two samples of each organ collected per treatment group were fixed in 10% neutral buffered formalin and refrigerated for subsequent histological analysis. The other samples were quick frozen in liquid nitrogen and then transferred to a -80°C freezer. The femurs were placed in sealed plastic bags and stored in a -20°C freezer.

For analysis, femurs collected from all treatments were allowed to thaw overnight in a standard refrigerator. Excess muscle tissue was removed. A femur from each bird was weighed and the wet femur weight was recorded and relative wet femur weight was calculated as a percent of body weight. Femurs were then placed in a drying oven for 48 hours at 50°C. Estrogen has been shown to have an effect on the trabicular bone and the amount of bone marrow in the bone. The weight difference between the wet femur

weight and the dry femur weight would indicate the relative amount of bone minerals vs. bone marrow present in the starting bone.

Once removed from the drying oven, the femurs were placed in a dessicator and allowed to cool to room temperature. The dry weights of the femurs were recorded and the relative dry femur weights were calculated as a percent of body weight. The femurs were then placed in crucibles and ashed for 18 hours at 600°C. The femurs were ashed to determine if any of the treatments had an effect on the mineral content of the femur through medullary bone induction. Medullary bone is the part of the femur that the adult chicken uses to store calcium for eggshell production. Once removed from the oven, the femurs were placed in a dessicator and allowed to cool to room temperature. The ashed femur weights were recorded and relative ashed femur weights were calculated as a percent of body weight.

The amount of genistein contained in the plasma of the birds was determined by high performance liquid chromatography (HPLC) (Appendix 5). This protocol was modified from the method created by Thomas et al., 2001. Briefly, genistein in the plasma samples were hydrolyzed with *Helix pomatia* glucuronidase/sulfatase to allow for the measurement of total genistein. The samples were then extracted with ether, reconstituted, and subjected to HPLC analysis.

A protein analysis was performed on plasma samples to determine the amount of protein present in the plasma. The analysis was performed utilizing the Bio-Rad Protein Assay protocol (Appendix 2) (Bio-Rad Inc.).

An enzyme linked immunosorbent assay (ELISA) was performed using the sandwich elisa protocol (Appendix 4) to determine amount of vitellogenin protein in plasma samples. The primary capture antibody was polyclonal rabbit anti-sea bream

vitellogenin diluted 1:1000 and the primary detection antibody was monoclonal mouse anti-bird vitellogenin (Biosense Laboratories, Norway), diluted 1:1000. The ELISA was performed with the VECTASTAIN Elite ABC Kit (Mouse IgG) and was visualized with the ABTS Substrate Kit (Vector Labs, Burlingame, CA). The absorbance was read on a microplate reader at 405 nm. The positive control thr this ELISA was plasma from adult hens confirmed to be laying eggs. Plasma vitellogenin concentration was expressed as a percent of control.

Statistical relationships were evaluated using SAS statistical software (SAS Institute, 2002). One-way ANOVA and Tukey's Studentized Range (HSD) Test were conducted to determine any statistical differences. Statistical differences were determined to be significant at a P value of 0.05 or less. All percentage data were subjected to arc sine square root transformation prior to analysis.

Results and Discussion

Broiler chickens grown to 14 days will typically have consumed approximately 500 g of feed with 35% of that being soybean meal, which contains essentially all of the isoflavones or isoflavone glycosides present in the starting soybeans (Eldridge and Kwolek, 1983). Genistein has been shown in the literature to have an estrogenic equivalency of about 1/1200th the potency of estradiol (Reinli and Block, 1996, Korach et al., 1997). This would be equivalent to the average broiler chicken consuming about 0.036 mg of estradiol over 14 days. Levels of estrogen exposure similar to this have been reported to produce significant effects on reproductive development in mammals (Levy et

al., 1995). However, the isoflavone content of soybeans can vary greatly from crop to crop.

The total amount of genistein given to birds during the 14-day trial for the G10 and G40 treatment groups was 140 mg, and 560 mg, respectively. These amounts, expressed as estrogen units, were equal to approximately 0.008 mg and 0.033 mg of estradiol daily. Over the 14 days of treatment, it would be equal to 0.112 mg and 0.462 mg of estradiol, respectively, with these values being within the upper range of possible dietary exposure of chickens in commercial production.

There were no significant differences (P= 0.5759) in the starting body weight for any of the treatments (Table 13). This was expected since the birds were all obtained from the same hatchery at the same time. Birds that received the SV treatment had significantly higher body weights for days 5 (P= 0.0012), 10 (P< 0.0001), and 15 (P< 0.0001) (Tables 13 and 14) than all other treatments. Birds that received the SV treatment also had significantly higher body weight gains for days 0 to 5 (P= 0.0007), 5 to 10 (P< 0.0001), and 10 to 15 (P< 0.0001) than all other treatments (Tables 15 and 16). These differences in body weight and weight gain can be attributed to the nutritional differences in the two diets (Tables 11 and 12). The standard diet supplied more crude protein and calories to the birds than the control diet. This difference is due to the difficulty in creating a diet that utilizes egg white as compared to the typical corn/soymeal diet. The standard diet may also have been more palatable and those birds could have eaten more than the birds on the control diet.

There were no significant differences (P= 0.1365) in the actual liver weights due to the treatments (Table 17). Birds receiving the SV treatment had significantly lower (P< 0.0001) relative liver weights (Table 17) than other treatments. This is due to the

differences in day 15 body weights (Table 14). The liver is the site of synthesis of lipoproteins deposited in the egg yolk. Exposure to estrogen would cause the liver to produce these lipoproteins, including vitellogenin. Differences in liver weights based on the treatments would indicate that the livers of the birds were producing these lipoproteins.

Estrogen exposure causes the oviduct to grow in both size and amount of glandular tissue. Birds that received the DES treatment had significantly higher oviduct weights ($P < 0.0001$) and relative oviduct weights ($P < 0.0001$) as compared to all other treatments (Table 18). The effect of DES on the oviduct was expected since it is such a strong estrogen agonist in the chicken. Genistein exposure produced an increasing trend in oviduct weight with increasing dose. However, this trend did not achieve significance.

Birds receiving the SV treatment had significantly higher wet femur weights ($P < 0.0001$) than the other treatments, due to their larger body size (Table 19). The G40 and DES treatments had the highest relative wet femur weights, but there were no statistical differences ($P = 0.0721$) between any treatments (Table 19).

Birds receiving the SV treatment had significantly higher dry femur weights ($P < 0.0001$) after 48 hours at 50°C than all other treatments (Table 20). The difference in dry femur weights is likely due to the SV treatment having higher final body weights (Table 15). The DES treatment had the second lowest dry femur weight, but the highest relative dry femur weight. There were no significant differences ($P = 0.8383$) when comparing the relative dry femur weights (Table 20). These results suggest that estrogen has no effect on the amount of bone marrow in the femur of immature chickens at this age.

Birds that recieved the SV treatment had significantly higher ashed femur weights ($P = 0.0087$) after 18 hours at 600°C than all other treatments (Table 21). This difference

in ashed femur weights is most likely due to the differences in the final body weights (Table 15). The DES treatment had the second lowest ashed femur weight, but the second highest relative ashed femur weight (Table 21). The SV treatment had the highest relative ashed femur weight (Table 21). It was significantly higher ($P < 0.0001$) than the CV and G10 treatments, but not the G40 or DES treatments.

The SV treatment had significantly higher plasma protein content (mg/ml) ($P = 0.0163$) than the CV treatment (Table 22). There were no other significant differences between treatments.

The G40 treatment had the highest amount of genistein in the plasma (Table 23). The G10 treatment had the second highest amount. This was expected since these treatments were the only two that received genistein. There were no significant differences ($P = 0.1905$) between any of the treatment groups though (Table 23).

The results of this experiment show that DES is a very strong estrogen agonist in the female chick. The oral dose of genistein given did not show an estrogenic response in the female chick. This lack of response could be a result of too little genistein being absorbed into the body after it was metabolized in the gut.

Orally ingested genistein appears to have some estrogenic potency in the chick, as compared to a known, highly potent synthetic estrogen. In the chick oviduct model system, genistein induced the appearance of estrogen and progesterone receptors, increased cellular proliferation, and promoted the ability of progesterone to induce ovalbumin synthesis. However, at the doses used in this study, genistein did not induce significant growth of the oviduct as did DES, nor did it significantly increase plasma vitellogenin, which is a common marker for estrogenic exposure in a number of species. Thus, it appears that ingested genistein is only weakly estrogenic in the chick.

The relatively weak effects of genistein as compared to DES could be due to a number of factors. Genistein is poorly absorbed from the intestine of most animals, is subject to bacterial conversion, and is rapidly metabolized in the hepatic circulation and conjugated to bile for excretion. Furthermore, it is possible that genistein does not possess the full range of activity of steroidal estrogens. Many estrogenic substances exhibit both estrogen agonist and antagonist activities through competition for receptors and plasma binding proteins. This may have been the case with genistein in this study. It is also possible that more pronounced estrogenic effects would have been induced with a longer duration of dosing. It is possible that the chicken reproductive system may be more responsive to genistein during a different period of development.

TABLE 11. Ingredient percentages and calculated analysis of broiler starter diet with a low level of isoflavones (control diet).

Ingredient	(%)
Corn	78.65
Egg Albumin (82% CP)	13.23
Soybean Meal (48% CP)	3.00
Rice Mill Feed	1.38
Dicalcium Phosphate[1]	1.37
Limestone (38% Ca)	1.35
Vitamin Premix[2]	1.00
L-Lysine	0.25
Trace Mineral Premix[3]	0.25
Salt	0.02
Calculated Analysis	
Crude Protein (%)	18.15
Fat	3.101
Fiber	2.542
ME (kcal/kg)	2870.00
Calcium (%)	0.95
Available Phosphorus (%)	0.45
Methionine (%)	0.60
Methionine & Cystein (%)	1.00
Lysine (%)	1.15
Arginine (%)	1.14
Threonine (%)	0.82

[1] Contains 18.5% phosphorous and 24.1% calcium

[2] Supplied the following per kg of complete feed: vitamin A, 32,000 IU (retinyl palmitate); cholecaliciferol, 8,000 IU; vitamin E, 32 IU (di-tocopheryl acetate); menadione, 8 mg; riboflavin, 22 mg; pantothenic acid, 52 mg; niacin, 144 mg; choline, 2000 mg; vitamin B_{12}, 0.08 mg; folic acid, 20 mg; thiamin, 4 mg; pyridoxine, 8.8 mg; biotin, 0.2 mg, ethoxyquin, 500 mg.

[3] Supplied the following per kg of complete feed: manganese, 125 mg; iodine, 1 mg; iron, 55 mg; copper, 6 mg; zinc, 55 mg; selenium, 0.3mg.

TABLE 12. Ingredient percentages and calculated analysis of standard broiler starter diet (standard diet).

Ingredient	(%)
Corn	52.79
Soybean Meal (48% CP)	37.20
Poultry Oil	5.08
Alfalfa Meal	1.00
Dicalcium Phosphate[1]	1.97
Limestone (38% Ca)	0.84
Vitamin Premix[2]	0.25
DL-Methionine	0.20
Trace Mineral Premix[3]	0.25
Salt	0.42
Calculated Analysis	
Crude Protein (%)	22.97
Fat	7.40
Fiber	2.85
ME (kcal/kg)	3120.12
Calcium (%)	0.87
Available Phosphorus (%)	0.51
Methionine (%)	0.57
Methionine & Cystein (%)	0.93
Lysine (%)	1.32
Arginine (%)	1.64
Threonine (%)	0.92

[1] Contains 18.5% phosphorous and 24.1% calcium

[2] Supplied the following per kg of complete feed: vitamin A, 16,000 IU (retinyl palmitate); cholecaliciferol, 4,000 IU; vitamin E, 16 IU (di-tocopheryl acetate); menadione, 4 mg; riboflavin, 11 mg; pantothenic acid, 26 mg; niacin, 72 mg; choline, 1000 mg; vitamin B_{12}, 0.04 mg; folic acid, 10 mg; thiamin, 2 mg; pyridoxine, 4.4 mg; biotin, 0.1 mg, ethoxyquin, 250 mg.

[3] Supplied the following per kg of complete feed: manganese, 125 mg; iodine, 1 mg; iron, 55 mg; copper, 6 mg; zinc, 55 mg; selenium, 0.3 mg.

TABLE 13. Average body weight per bird on days 0 and 5.

Treatment	d 0 (g)	P value	N	d 5 (g)	P value	N
SV	36.4 ± 2.3 [NS]	0.5759	20	91.8 ± 12.4 [a]	0.0012	19
CV	35.2 ± 3.5 [NS]		20	82.9 ± 7.8 [b]		20
G10	35.0 ± 2.7 [NS]		20	82.0 ± 9.1 [b]		19
G40	35.5 ± 3.1 [NS]		20	80.6 ± 9.4 [b]		20
DES	35.9 ± 2.7 [NS]		20	79.1 ± 10.0 [b]		20

[a-b] Values in columns not followed by the same superscript differ significantly (P≤0.05).
[NS] Values do not differ significantly (P≤0.05).
[1] Values shown are treatment averages with standard deviation.

TABLE 14. Average body weight per bird on days 10 and 15.

Treatment	d 10 (g)	P value	N	d 15 (g)	P value	N
SV	210.7 ± 31.5 [a]	<0.0001	18	398.6 ± 54.6 [a]	<0.0001	18
CV	150.1 ± 19.8 [b]		20	241.1 ± 25.4 [b]		16
G10	156.9 ± 20.5 [b]		19	239.2 ± 30.3 [b]		11
G40	154.8 ± 18.5 [b]		20	244.1 ± 25.2 [b]		15
DES	151.3 ± 18.2 [b]		20	229.8 ± 25.3 [b]		13

[a-b] Values in columns not followed by the same superscript differ significantly (P≤0.05).
[NS] Values do not differ significantly (P≤0.05).
[1] Values shown are treatment averages with standard deviation.

TABLE 15. Average body weight gain per bird per period for days 0 to 5 and 5 to 10.

			Gain per period (g)			
Treatment	0 to 5 d	P value	N	5 to 10 d	P value	N
SV	55.4 ± 12.7 [a]	0.0007	19	117.7 ± 23.2 [a]	<0.0001	18
CV	47.7 ± 5.4 [b]		20	67.3 ± 16.0 [b]		20
G10	47.1 ± 8.1 [b]		19	74.9 ± 13.3 [b]		19
G40	45.1 ± 7.7 [b]		20	74.2 ± 11.8 [b]		20
DES	43.3 ± 9.2 [b]		20	72.1 ± 12.5 [b]		20

[a-b] Values in columns not followed by the same superscript differ significantly (P≤0.05).
[NS] Values do not differ significantly (P≤0.05).
[1] Values shown are treatment averages with standard deviation.

TABLE 16. Average body weight gain per bird per period for days 10 to 15 and 0 to 15.

Treatment	Gain per period (g) 10 to 15 d	P value	N	0 to 15 d	P value	N
SV	187.9 ± 26.6^{a}	<0.0001	18	362.0 ± 55.2^{a}	<0.0001	18
CV	89.4 ± 19.4^{b}		16	205.2 ± 23.5^{b}		16
G10	84.5 ± 18.4^{b}		11	203.7 ± 30.4^{b}		11
G40	91.9 ± 19.6^{b}		15	208.5 ± 23.4^{b}		15
DES	81.5 ± 17.1^{b}		13	193.3 ± 24.4^{b}		13

$^{a-b}$ Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
^{NS} Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.

TABLE 17. Weight and relative weight of the liver at 15 days of age.

Treatment	Liver weight (g)	P value	Relative weight (%)	P value	
SV	13.75 ± 2.00^{NS}	0.1365	3.46 ± 0.28^{b}	<0.0001	18
CV	12.27 ± 1.46^{NS}		5.11 ± 0.52^{a}		16
G10	12.00 ± 3.05^{NS}		4.97 ± 0.83^{a}		11
G40	12.64 ± 2.09^{NS}		5.16 ± 0.53^{a}		15
DES	12.08 ± 2.14^{NS}		5.24 ± 0.64^{a}		13

$^{a-b}$ Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
^{NS} Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from d 15 in Table 13.

TABLE 18. Weight and relative weight of the oviduct at 15 days of age.

Treatment	Oviduct weight (g)	P value	Relative weight (%)	P value	N
SV	0.058 ± 0.010^{a}	<0.0001	0.015 ± 0.003^{a}	<0.0001	18
CV	0.041 ± 0.010^{a}		0.017 ± 0.004^{a}		16
G10	0.039 ± 0.012^{a}		0.016 ± 0.004^{a}		11
G40	0.039 ± 0.012^{a}		0.016 ± 0.005^{a}		15
DES	0.195 ± 0.092^{b}		0.086 ± 0.041^{b}		13

$^{a-b}$ Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
^{NS} Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from d 15 in Table 13.

TABLE 19. Weight and relative weight of one femur from each bird in each treatment at 15 days of age.

Treatment	Wet Femur Weight (g)	P value	Relative Weight (%)	P value	N
SV	2.172 ± 0.092 [a]	<0.0001	0.543 ± 0.012 [NS]	0.0721	18
CV	1.302 ± 0.052 [b]		0.539 ± 0.014 [NS]		16
G10	1.333 ± 0.070 [b]		0.557 ± 0.019 [NS]		11
G40	1.441 ± 0.055 [b]		0.591 ± 0.018 [NS]		15
DES	1.328 ± 0.050 [b]		0.579 ± 0.016 [NS]		13

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from d 15 in Table 13.

TABLE 20. Weight and relative weight of one femur from each bird in each treatment at 15 days of age after 48 hours at 50°C.

Treatment	Dry Femur Weight (g)	P value	Relative Weight (%)	P value	N
SV	0.828 ± 0.035 [a]	<0.0001	0.207 ± 0.004 [NS]	0.8383	18
CV	0.487 ± 0.017 [b]		0.202 ± 0.005 [NS]		16
G10	0.478 ± 0.021 [b]		0.200 ± 0.007 [NS]		11
G40	0.502 ± 0.019 [b]		0.206 ± 0.006 [NS]		15
DES	0.479 ± 0.018 [b]		0.209 ± 0.006 [NS]		13

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from d 15 in Table 13.

TABLE 21. Weight and relative weight of one femur from each bird in each treatment at 15 days of age after 18 hours at 600°C.

Treatment	Ashed Femur Weight (g)	P value	Relative Weight (%)	P value	N
SV	0.297 ± 0.014 [a]	0.0087	0.074 ± 0.002 [a]	<0.0001	18
CV	0.155 ± 0.006 [b]		0.064 ± 0.004 [b]		16
G10	0.147 ± 0.010 [b]		0.062 ± 0.004 [b]		11
G40	0.158 ± 0.006 [b]		0.065 ± 0.002 [ab]		15
DES	0.154 ± 0.007 [b]		0.067 ± 0.003 [ab]		13

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from d 15 in Table 13.

TABLE 22. Amount of protein and vitellogenin contained in the plasma of birds at 15 days of age.

Treatment	Protein (mg/ml)	P value	N	Vitellogenin[2]	P value	N
SV	24.111 ± 0.520 [a]	0.0163	18	112.5 ± 0.010 [ab]	0.0264	12
CV	19.477 ± 0.910 [b]		16	100.0 ± 0.007 [b]		11
G10	21.500 ± 1.030 [ab]		11	200.0 ± 0.007 [ab]		3
G40	22.124 ± 1.162 [ab]		15	250.0 ± 0.011 [a]		7
DES	21.943 ± 1.279 [ab]		13	87.5 ± 0.004 [ab]		5

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Expressed as a percent of the control (CV) and calculated to represent the total protein.

TABLE 23. Amount of genistein contained in the plasma of birds at 15 days of age.

Treatment	Integrated Curve Area	P value	Genistein (μg/ml)	N
SV	9.936 ± 2.220 [NS]	0.1905	0.3016	4
CV	12.447 ± 0.769 [NS]		0.3686	4
G10	12.526 ± 3.291 [NS]		0.3707	4
G40	15.450 ± 3.877 [NS]		0.4485	4
DES	11.736 ± 3.243 [NS]		0.3496	4

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.

VI. MANUSCRIPT 3

THE EFFECTS OF SHORT-TERM INJECTED EXPOSURE TO THE ISOFLAVONE GENISTEIN ON THE IMMATURE FEMALE BROILER CHICKENS

Abstract

To determine the effects of short-term injected exposure to the estrogenic soy isoflavone genistein on the immature female chick; 50 chicks were wing banded, individually weighed, and placed into battery brooders. Chicks were assigned to five treatment groups with 10 birds in each group. Each treatment group received a daily injection of either 0.2 ml sesame oil vehicle (SV), (CV); 1 mg diethylstilbestrol (DES); 10 mg genistein (G10); or 40 mg genistein (G40). The CV, G10, G40, and DES groups were fed an egg-white based chick starter diet. The SV group was fed a standard chick starter diet. The birds were killed at 15 days of age; blood, oviducts, livers, and femurs were collected. DES treatment increased oviduct weight and relative oviduct weight as compared to all other treatments ($P<0.05$). The SV treatment increased body weight and weight gain, and decreased relative liver weight ($P<0.05$) as compared to the other treatments. The G40 and DES treatments showed adult hen behaviors, marked development the right oviduct, and increased the absolute oviduct weight and oviduct weight as a percentage of final body weight as compared to the CV and G10 treatments

The DES treatment showed partially developed right oviducts and the G40 treatment showed membranous cystic right oviducts. These results indicate that genistein acts as a weak estrogen with selective effects on the chick oviduct.

Introduction

Estrogens are endogenous steroid hormones with numerous physiological actions (Norris, 1985; van Tienhoven, 1983; Korach et al., 1997; Bennink and Boerrigter, 2003). They are members of the steroid hormone family that also contains androgens, and progestagens (Saunders, 1998). 17β-estradiol is the predominant estrogen in animals and is mainly secreted by the ovaries (Bennink and Boerrigter, 2003). Estrogens are involved in the development and function of the male and female genital tract (Sonnenschein and Soto, 1998). They are also involved in the development of female secondary sexual characteristics (Norris, 1985; van Tienhoven, 1983).

Estrogenic compounds come from a number of different sources. Many of them are naturally occurring compounds synthesized by plants, natural steroidal estrogens or synthetic estrogens used in medicine that were excreted into the environment, or chemical that were synthesized for another purpose and then found to have estrogenic activity (Burton and Wells, 2002). Diethylstilbestrol was one of the first non-steroidal estrogens to be synthesized and has very strong estrogenic activity (Bennink and Boerrigter, 2003). It has been shown that exposure to exogenous hormones during fetal development can profoundly alter the sexual development in many animals (McLachlan and Newbold, 1987).

Phytoestrogens are plant chemicals that resemble natural estrogens in either their structure or function (Whitten and Naftolin, 1998; Bradbury and White, 1954). They

have the ability to mimic the actions of endogenous estrogens. They can act as estrogen agonists or antagonists. Phytoestrogens can also alter the pattern of synthesis and/or metabolism for the endogenous hormones (Sonnenschein and Soto, 1998; Whitten et al., 1995).

The largest class of phytoestrogens is the isoflavone class. This class includes genistein, genistin, daidzein, biochanin A, formononetin, and pratensein (Thomas, 1997). Isoflavones are low molecular weight diphenolic compounds. They often serve as antimicrobial compounds (phytoalexins) that are synthesized de novo in response to the plant's exposure to a pathogen (Burton and Wells, 2002). Genistein, daidzein and glycitein are the major isoflavones found in soybeans. They are generally found at a ratio of approximately 1.3:1.0:0.2 in soybeans (LC Laboratories, 2004). Isoflavone concentrations in soybeans can vary from year to year and from place to place. The isoflavone content of soybeans can vary by year and variety by a factor of 3-5 (Franke et al., 1994).

In the animal gut, the naturally occurring glycosides are hydrolyzed into aglylcones (Barrett, 1996; Day et al., 2000). The aglycones and their metabolites are then excreted, metabolized bacterially in the gut, or absorbed into the blood (Barrett, 1996). Genistein and biochanin A are usually broken down by microbial activity in the digestive system (Adams, 1995). Genistein is naturally deglycosylated and absorbed into the blood stream as 7-O-β-glucuronide, not as genistein (Bennetau-Pelissero et al., 2001; Sfakianos et al., 1997; Yuan et al., 2003). Both genistein and 7-O-β-glucuronide are not very well absorbed from the intestine. They are very efficiently extracted from the portal blood in the liver and then excreted into the bile in a conjugated form (sulfate ester or

glucuronide) (Sfakianos et al., 1997). Due to this efficient enterohepatic circulation genistein may accumulate within the enterohepatic circuit or may be excreted with a long half-life (Sfakianos et al., 1997).

Genistein shares structural features with the potent estrogen 17-β estradiol. It is particularly similar in the phenolic ring and the distance between its 4'-and 7'- hydroxyl groups (Dixon et al., 2002). These features allow genistein to bind to the estrogen receptors in animals (Hopert et al., 1998; Milligan et al., 1998; Messina et al., 1994; Makarevich et al., 1997). The ligand:receptor complex it creates induces the expression of estrogen-responsive genes that can result in an increased uterine mass, among other effects (Santell et al., 1997; Hopert et al., 1998; Milligan et al., 1998). Genistein or its metabolites may function as partial agonists at the chicken estrogen receptor with regard to the stimulation of expression of estrogen responsive genes (Ratna, 2002). Genistein, due to its lower potency as compared to estrogen, functions as a partial agonist at the chicken estrogen receptor. At certain ratios, it can block the activity of estrogen (Ratna, 2002).

Estrogen is a pivotal factor involved in the development of sexual differentiation, female secondary sexual characteristics, and vitellogenesis in birds (van Tienhoven, 1983). Posthatch estrogen treatment is known to alter development of the oviduct anatomy of chicks (Kohler et al., 1969; Oka and Schimke, 1969). The administration of exogenous estrogen to the immature chick has been shown to result in impressive increases in oviduct size and weight. These are proportional to the amount of hormone given (Oka and Schimke, 1969, Hertz and Tulner, 1947; Munro and Kosin, 1943). Repeated administration of estrogen to the immature female chick results in rapid cell proliferation and formation of tubular gland cells. It also causes specific egg white

proteins such as lysozyme and ovalbumin to appear in the oviduct (Oka and Schimke, 1969)

The literature contains very few reports on the estrogenicity of isoflavones in poultry species. Considering that developmentally inappropriate estrogen exposures cause significant abnormalities in mammals and birds, it is important to determine whether naturally occurring estrogenic compounds prevalent in commercial poultry diets alter reproductive development and subsequent reproductive efficiency of domestic poultry. The objective of the study was to examine the estrogenicity of the soy isoflavone genistein in the immature chicken through stimulation of the growth response of the chick oviduct and liver, amount of genistein, vitellogenin, and protein present in the plasma, and through deposition of medullary bone in the femur.

Materials and Methods

For this experiment, a total of 50 female commercial strain broiler chicks were obtained from a commercial hatchery. Ten birds were randomly assigned to each of five battery brooder cages as treatment groups. The birds were housed in an environmentally controlled room and maintained at a room temperature of 31°C (88°F) with continuous light. A small chick waterer was provided for the first five days, after that trough waterers and feeders were used. Management and experimental treatment of the chicks was approved by the Auburn University Instututional Animal Care and Use Committee.

As appropriate for the assigned treatment group, birds were injected daily for 14 days with one of five different treatments. The treatments were 1 mg diethylstilbestrol (DES) as a positive estrogen control; 10 mg genistein (G10); 40 mg genistein (G40); control vehicle (CV) as a negative control; or standard vehicle (SV) as a comparison to

commercial chickens. All treatments were dissolved in a 0.2 ml sesame oil vehicle. Daily dosing was carried out using a 1 ml tuberculin syringe and a 21-gauge, 1 in. needle. All injections were given in the subcutaneous tissue in the back of the neck.

Birds in the CV, G10, G40, and DES treatments were fed a*d libitum* a pelleted and crumbled corn/egg white starter diet (Table 24) that was devoid of isoflavones. The diet had a crude protein content of 18.5% and supplied 2870 Kcal/Kg. The egg white used in the diet was freeze-dried raw egg white. Since raw egg white contains avidin, which binds biotin, extra vitamin premix was added to the diet to prevent a biotin deficiency. Birds in the SV treatment were fed a*d libitum* a standard chick starter diet (Table 25). The diet had a crude protein content of 23% and supplied 3120 Kcal/Kg.

On days 0, 5, 10, and 15, the body weights of all birds were recorded and the gain per bird was calculated for days 0 to 5, 5 to 10, and 10 to 15. On day 15, blood was collected from the ulnar vein into heparinized tubes and stored on ice. Tubes of blood were centrifuged at 1500 RPM for fifteen minutes. Plasma was collected, aliquoted into tubes and stored in a -20°C freezer.

Birds were euthanized by CO_2 asphyxiation and necropsied on the day 15. Liver, oviduct, and both femurs were collected from each bird and were weighed. Relative weight was calculated as a percent of final body weight. Two samples of each organ collected per treatment group were fixed in 10% neutral buffered formalin and refrigerated for subsequent histological analysis. The other samples were quick frozen in liquid nitrogen and then transferred into a -80°C freezer. The femurs were placed in sealed plastic bags and stored in a -20°C freezer.

For analysis, femurs collected from all treatments were allowed to thaw overnight in a standard refrigerator. Any excess muscle tissue was removed. A femur from each bird was weighed and the wet femur weight was recorded and relative wet femur weight was calculated as a percent of body weight. Femurs were then placed in a drying oven for 48 hours at 50°C. Estrogen has been shown to have an effect on wet weight through effects on the amount of bone marrow in the bone. The difference in weight between the wet femur weight and the dry femur weight would be an index of the amount of blood marrow present in the starting bone.

Once removed from the drying oven, the femurs were placed in a dessicator and allowed to cool to room temperature. The dry weights of the femurs and the relative dry femur weights were recorded. The femurs were then placed in crucibles and ashed for 18 hours at 600°C. The femurs were ashed to determine if any of the treatments had an effect on the mineral content, and by implication, the medullary bone in the femur. Medullary bone is the part of the femur that the adult chicken uses to store calcium for eggshell production. Once removed from the oven, the femurs were placed in a dessicator and allowed to cool to room temperature. The ashed femur weights and relative ashed femur weights were calculated and recorded.

The amount of genistein contained in the plasma of the birds was determined by high performance liquid chromatography (HPLC) (Appendix 5). This protocol was modified from the method created by Thomas et al., 2001. Briefly, genistein in the plasma samples were hydrolyzed with *Helix pomatia* glucuronidase/sulfatase to allow for the measurement of total genistein. The samples were then extracted with ether, reconstituted, and subjected to HPLC analysis.

A protein analysis was performed on plasma samples utilizing the Bio-Rad Protein Assay protocol (Appendix 2) to determine the amount of protein in the plasma (Bio-Rad Inc.).

An enzyme linked immunosorbent assay (ELISA) was performed using the sandwich elisa protocol (Appendix 4) to determine amount of vitellogenin protein in plasma samples. The primary capture antibody was polyclonal rabbit anti-sea bream vitellogenin diluted 1:1000 and the primary detection antibody was monoclonal mouse anti-bird vitellogenin (Biosense Laboratories, Norway), diluted 1:1000. The ELISA was performed with the VECTASTAIN Elite ABC Kit (Mouse IgG) and was visualized with the ABTS Substrate Kit (Vector Labs, Burlingame, CA). The absorbance was read on a microplate reader at 405 nm. The positive control for this ELISA was plasma from adult hens confirmed to be laying eggs. Plasma vitellogenin concentration was expressed as a percent of control.

Statistical relationships were evaluated using SAS statistical software (SAS Institute, 2002). One-way ANOVA and Tukey's Studentized Range (HSD) Test were conducted to determine any statistical differences. Statistical differences were determined to be significant at a P value of 0.05 or less. All percentage data were subjected to arc sine square root transformation prior to analysis.

Results and Discussion

Over 14 days, broiler chickens will typically have consumed approximately 500 g of feed with 35% of that being soybean meal that contains essentially all of the isoflavones or isoflavone glycosides present in the starting soybeans (Eldridge and

Kwolek, 1983). The isoflavone content ofsoybeans can vary widely from crop to crop. Genistein has been shown in the literature to have an estrogenic equivalency of about 1/1200th the potency of estradiol (Reinli and Block, 1996, Korach et al., 1997). This would be equivalent to the average broiler chicken consuming about 0.036 mg of estradiol over 14 days. Levels of estrogen exposure similar to this have been reported to produce significant effects on reproductive development in mammals (Levy et al., 1995).

The total amount of genistein given to birds during the 14-day trial for the G10 and G40 treatment groups was 140 mg, and 560 mg, respectively. These amounts, expressed as estrogen units, were equal to approximately 0.008 mg and 0.033 mg of estradiol daily. Over the 14 days of treatment, it would be equal to 0.112 mg and 0.462 mg of estradiol, respectively, with these values being within the upper range of possible dietary exposure of chickens in commercial production.

There were no significant differences between any of the treatments for starting body weight (P= 0.7100) or for the day 5 body weight (P= 0.1147) (Table 26). Birds that received the SV treatment had significantly higher body weights for days 10 (P< 0.0001) and 15 (P< 0.0001) than all other treatments (Table 27). There were no significant differences (P= 0.0774) in the body weight gains for days 0 to 5 (Table 28). The SV treatment had significantly higher weight gains for days 5 to 10 (P< 0.0001) and 10 to 15 (P< 0.0001) than all other treatments (Tables 28 and 29). These differences in body weight and weight gain can be attributed to the nutritional differences in the two diets (Tables 24 and 25). The standard diet supplied more crude protein and calories to the birds than the control diet. This difference is due to the difficulty in creating a diet that utilizes egg white as compared to the typical corn/soymeal diet. The standard diet may

also have been more palatable and those birds could have eaten more than the birds on the control diet.

Birds receiving the DES treatment had significantly higher liver weights ($P < 0.0001$) and relative liver weights ($P < 0.0001$) than all other treatments (Table 30). Birds receiving the SV treatment had significantly lower liver weights ($P < 0.0001$) and relative liver weights ($P < 0.0001$) than all of the other treatments (Table 30). The liver is the site of synthesis of lipoproteins deposited in the egg yolk. Exposure to estrogen causes the liver to produce these lipoproteins, including vitellogenin. Differences in liver weights based on the treatments would show that the livers of the birds were preparing to or producing these lipoproteins. The livers of the birds receiving the DES treatment had very fatty livers. This is most likey due to the livers of these birds preparing to produce or producing lipoproteins for egg production.

Exposure to estrogen causes the oviduct to grow in both size and amount of glandular tissue. Birds that received the DES treatment had significantly higher ($P < 0.00001$) oviduct weights and relative oviduct weights ($P < 0.0001$) than all other treatments (Table 31). Birds in the G40 treatment had significantly higher ($P < 0.0001$) oviduct weights and relative oviduct weights ($P < 0.0001$) than the CV and G10 treatments (Table 31). Birds in the G40 treatment showed membranous cystic right oviducts and the birds receiving the DES treatment showed partially developed right oviducts.

Birds in the SV treatment had significantly higher ($P < 0.0001$) wet femur weights than all of the other treatments (Table 32) most likely due to the differences in final body weights (Table 27). DES treated birds had significantly lower wet femur weights ($P < 0.0001$) and relative wet femur weights ($P < 0.0001$) than all of the other treatments

(Table 32). The increasing doses of genistein tended to have a decreasing effect on the wet femur weights.

The SV treatment had significantly higher dry femur weights (P< 0.0001) after 48 hours at 50°C than all other treatments (Table 33). Birds in the DES treatment had significantly lower (P< 0.0001) dry femur weights than the other treatments (Table 33). The SV treatment birds had significantly higher (P< 0.0001) relative dry femur weights than the CV, DES, and G40 treatments (Table 33). Birds in the DES treatment had significantly lower (P< 0.0001) relative dry femur weights than all of the other treatments (Table 33). Estrogen has an effect on the amount of bone marrow located in the bones of animals. Birds in the DES treatment, a strong synthetic estrogen, had lower dry femur weights and relative dry femur weights than all of the other treatment. This suggests that the DES treatment had a decreasing effect on the amount of bone marrow in the femur.

The SV treatment had significantly higher (P< 0.0001) ashed femur weights after 18 hours at 600°C than all other treatments (Table 34). This was most likely due to the final body weight (Table 27). Birds in the DES treatment had significantly lower (P< 0.0001) ashed femur weights than the SV and G10 treatments (Table 34). The SV treatment birds had significantly higher (P< 0.0001) relative ashed femur weights than the CV, G40, and DES treatments (Table 34). The DES treatment had significantly lower (P< 0.0001) relative ashed femur weights than the SV and G10 treatments (Table 34).

Birds in the DES treatment had significantly higher (P< 0.0001) plasma protein contents than all other treatments (Table 35). The G40 treated birds had significantly lower (P< 0.0001) plasma protein content than the SV and DES treatments (Table 35).

Birds in the G40 treatment had significantly higher (P< 0.0001) amounts of genistein in the plasma than all other treatments (Table 36). The G10 treated birds had

significantly higher ($P < 0.0001$) plasma genistein levels than all treatments other than G40 (Table 36). The birds in the DES treatment had significantly lower ($P < 0.0001$) plasma genistein contents than all other treatments except the CV treatment (Table 36). The G40 and G10 treated birds were the only ones that were given genistein. It makes sense that these treatments would have the highest plasma genistein levels. Birds in the SV treatment were exposed to genistein through the soybean meal in the standard diet. These birds showed higher plasma genistein levels than the DES and CV treated birds that were not exposed to genistein.

Throughout the course of treatment, the birds in the DES and G40 treatments showed signs of adult hen mating behaviors. This is consistant with the effects of estrogen treatment.

The genistein treatments did not seem to have an effect on the livers of the birds. The DES treatment did have an effect and caused an increase in the liver weight. Both compounds had an effect on oviduct weights. This difference in estrogenic effects suggests that genistein is a selective estrogen agonist in the female chick.

TABLE 24. Ingredient percentages and calculated analysis of broiler starter diet with a low level of isoflavones (control diet).

Ingredient	(%)
Corn	78.65
Egg Albumin (82% CP)	13.23
Soybean Meal (48% CP)	3.00
Rice Mill Feed	1.38
Dicalcium Phosphate[1]	1.37
Limestone (38% Ca)	1.35
Vitamin Premix[2]	1.00
L-Lysine	0.25
Trace Mineral Premix[3]	0.25
Salt	0.02
Calculated Analysis	
Crude Protein (%)	18.15
Fat	3.101
Fiber	2.542
ME (kcal/kg)	2870.00
Calcium (%)	0.95
Available Phosphorus (%)	0.45
Methionine (%)	0.60
Methionine & Cystein (%)	1.00
Lysine (%)	1.15
Arginine (%)	1.14
Threonine (%)	0.82

[1] Contains 18.5% phosphorous and 24.1% calcium

[2] Supplied the following per kg of complete feed: vitamin A, 32,000 IU (retinyl palmitate); cholecaliciferol, 8,000 IU; vitamin E, 32 IU (di-tocopheryl acetate); menadione, 8 mg; riboflavin, 22 mg; pantothenic acid, 52 mg; niacin, 144 mg; choline, 2000 mg; vitamin B_{12}, 0.08 mg; folic acid, 20 mg; thiamin, 4 mg; pyridoxine, 8.8 mg; biotin, 0.2 mg, ethoxyquin, 500 mg.

[3] Supplied the following per kg of complete feed: manganese, 125 mg; iodine, 1 mg; iron, 55 mg; copper, 6 mg; zinc, 55 mg; selenium, 0.3mg.

TABLE 25. Ingredient percentages and calculated analysis of standard broiler starter diet (standard diet).

Ingredient	(%)
Corn	52.79
Soybean Meal (48% CP)	37.20
Poultry Oil	5.08
Alfalfa Meal	1.00
Dicalcium Phosphate[1]	1.97
Limestone (38% Ca)	0.84
Vitamin Premix[2]	0.25
DL-Methionine	0.20
Trace Mineral Premix[3]	0.25
Salt	0.42
Calculated Analysis	
Crude Protein (%)	22.97
Fat	7.40
Fiber	2.85
ME (kcal/kg)	3120.12
Calcium (%)	0.87
Available Phosphorus (%)	0.51
Methionine (%)	0.57
Methionine & Cystein (%)	0.93
Lysine (%)	1.32
Arginine (%)	1.64
Threonine (%)	0.92

[1] Contains 18.5% phosphorous and 24.1% calcium

[2] Supplied the following per kg of complete feed: vitamin A, 16,000 IU (retinyl palmitate); cholecaliciferol, 4,000 IU; vitamin E, 16 IU (di-tocopheryl acetate); menadione, 4 mg; riboflavin, 11 mg; pantothenic acid, 26 mg; niacin, 72 mg; choline, 1000 mg; vitamin B_{12}, 0.04 mg; folic acid, 10 mg; thiamin, 2 mg; pyridoxine, 4.4 mg; biotin, 0.1 mg, ethoxyquin, 250 mg.

[3] Supplied the following per kg of complete feed: manganese, 125 mg; iodine, 1 mg; iron, 55 mg; copper, 6 mg; zinc, 55 mg; selenium, 0.3 mg.

TABLE 26. Average body weight per bird on days 0 and 5.

Treatment	d 0 (g)	P value	N	d 5 (g)	P value	N
SV	40.1 ± 1.2 [NS]	0.7100	10	107.0 ± 2.7 [NS]	0.1147	10
CV	39.9 ± 0.9 [NS]		10	99.6 ± 3.3 [NS]		10
G10	39.8 ± 0.8 [NS]		10	98.9 ± 2.1 [NS]		10
G40	40.1 ± 1.1 [NS]		10	96.5 ± 3.5 [NS]		9
DES	41.5 ± 0.8 [NS]		10	96.6 ± 3.6 [NS]		10

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.

TABLE 27. Average body weight per bird on days 10 and 15.

Treatment	d 10 (g)	P value	N	d 15 (g)	P value	N
SV	249.9 ± 5.9 [a]	<0.0001	10	454.6 ± 37.0 [a]	<0.0001	10
CV	197.0 ± 6.6 [b]		8	354.2 ± 28.2 [b]		8
G10	211.1 ± 6.4 [b]		10	366.5 ± 33.2 [b]		10
G40	204.3 ± 6.4 [b]		9	371.2 ± 48.7 [b]		8
DES	197.5 ± 7.0 [b]		9	334.1 ± 35.1 [b]		9

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.

TABLE 28. Average body weight gain per bird per period for days 0 to 5 and 5 to 10.

	Gain per period (g)					
Treatment	0 to 5 d	P value	N	5 to 10 d	P value	N
SV	67.0 ± 1.8 [NS]	0.0774	10	142.8 ± 4.3 [b]	<0.0001	10
CV	59.7 ± 3.0 [NS]		10	100.9 ± 3.4 [a]		8
G10	59.0 ± 2.4 [NS]		10	112.3 ± 5.8 [a]		10
G40	56.3 ± 4.3 [NS]		9	107.6 ± 8.1 [a]		9
DES	55.1 ± 3.6 [NS]		10	101.5 ± 4.7 [a]		9

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.

TABLE 29. Average body weight gain per bird per period for days 10 to 15 and 0 to 15.

Treatment	Gain per period (g)					
	10 to 15 d	P value	N	0 to 15 d	P value	N
SV	205.6 ± 25.7^{b}	<0.0001	10	415.4 ± 34.4^{b}	<0.0001	10
CV	146.1 ± 35.8^{a}		8	302.2 ± 27.0^{a}		8
G10	155.4 ± 18.8^{a}		10	326.8 ± 33.7^{a}		10
G40	160.9 ± 32.3^{a}		8	324.8 ± 48.2^{a}		8
DES	137.6 ± 23.3^{a}		9	292.0 ± 35.4^{a}		9

$^{a-b}$ Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
^{NS} Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.

TABLE 30. Liver weight and relative liver weight at 15 days of age.

Treatment	Liver Weight (g)	P value	Relative Weight (%)	P value	N
SV	13.12 ± 0.959^{c}	<0.0001	2.89 ± 0.230^{c}	<0.0001	10
CV	19.20 ± 3.802^{b}		5.10 ± 0.539^{b}		8
G10	19.65 ± 4.244^{b}		5.33 ± 0.778^{b}		10
G40	19.70 ± 2.040^{b}		5.47 ± 0.816^{b}		8
DES	29.83 ± 4.165^{a}		7.83 ± 2.616^{a}		9

$^{a-b}$ Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
^{NS} Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from d 15 in Table 19.

TABLE 31. Oviduct weight and relative oviduct weight at 15 days of age.

Treatment	Oviduct weight (g)	P value	Relative Weight (%)	P value	N
SV	0.266 ± 0.080^{bc}	<0.0001	0.058 ± 0.017^{bc}	<0.0001	10
CV	0.128 ± 0.077^{c}		0.043 ± 0.032^{c}		8
G10	0.102 ± 0.030^{c}		0.051 ± 0.069^{c}		10
G40	0.509 ± 0.312^{b}		0.133 ± 0.071^{b}		8
DES	3.585 ± 0.384^{a}		1.079 ± 0.117^{a}		9

$^{a-b}$ Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
^{NS} Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from d 15 in Table 19.

TABLE 32. Wet femur weight and relative wet femur weight of one femur each bird in each treatment at 15 days of age.

Treatment	Wet Femur Weight (g)	P value	Relative Weight (%)	P value	N
SV	2.526 ± 0.337 [a]	<0.0001	0.553 ± 0.044 [a]	<0.0001	10
CV	2.010 ± 0.193 [b]		0.540 ± 0.046 [a]		8
G10	1.961 ± 0.259 [b]		0.534 ± 0.041 [a]		10
G40	1.909 ± 0.333 [b]		0.522 ± 0.046 [a]		9
DES	1.433 ± 0.171 [c]		0.429 ± 0.028 [b]		9

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from d 15 in Table 19.

TABLE 33. Dry femur weight and relative dry femur weight of one femur from each bird in each treatment at 15 days of age after 48 hours at 50°C.

Treatment	Dry Femur Weight (g)	P value	Relative Weight (%)	P value	N
SV	0.979 ± 0.000 [a]	<0.0001	0.214 ± 0.069 [a]	<0.0001	10
CV	0.707 ± 0.076 [b]		0.190 ± 0.017 [b]		8
G10	0.727 ± 0.095 [b]		0.198 ± 0.014 [ab]		10
G40	0.696 ± 0.101 [b]		0.191 ± 0.014 [b]		9
DES	0.546 ± 0.066 [c]		0.164 ± 0.012 [c]		9

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from d 15 in Table 19.

TABLE 34 Ashed femur weight and relative ashed femur weight of one femur from each bird in each treatment at 15 days of age after 18 hours at 600°C.

Treatment	Ashed Femur Weight (g)	P value	Relative Weight (%)	P value	N
SV	0.366 ± 0.052 [a]	<0.0001	0.080 ± 0.008 [a]	<0.0001	10
CV	0.239 ± 0.036 [bc]		0.064 ± 0.008 [bc]		8
G10	0.267 ± 0.039 [b]		0.073 ± 0.007 [ab]		10
G40	0.255 ± 0.043 [bc]		0.070 ± 0.007 [bc]		9
DES	0.208 ± 0.031 [c]		0.062 ± 0.007 [c]		9

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from d 15 in Table 19.

TABLE 35. Amount of protein and vitellogenin contained in the plasma of birds at 15 days of age.

Treatment	Protein (mg/ml)	P value	N	Vitellogenin [2]	P value	N
SV	22.215 ± 2.605 [b]	<0.0001	10	200.0 ± 0.002 [NS]	0.3785	4
CV	18.437 ± 6.342 [bc]		8	100.0 ± 0.003 [NS]		4
G10	17.784 ± 3.356 [bc]		10	150.0 ± 0.002 [NS]		8
G40	15.278 ± 4.156 [c]		9	200.0 ± 0.005 [NS]		3
DES	39.946 ± 4.015 [a]		9	50.0 ± 0.003 [NS]		5

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Expressed as a percent of the control (CV) and calculated to represent the total protein.

TABLE 36. Amount of genistein contained in the plasma of birds at 15 days of age.

Treatment	Integrated Curve Area	P value	Genistein (μg/ml)	N
SV	16.833 ± 5.194 [c]	<0.0001	0.4853	4
CV	11.492 ± 1.717 [cd]		0.3430	4
G10	30.356 ± 6.617 [b]		0.8460	4
G40	49.416 ± 14.551 [a]		1.3542	4
DES	6.669 ± 0.967 [d]		0.2145	4

[a-b] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[NS] Values do not differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.

VII. SUMMARY

Orally ingested genistein appears to have some estrogenic potency in the chick, as compared to a known, highly potent synthetic estrogen. In the chick oviduct model system, genistein induced the appearance of estrogen and progesterone receptors, increased cellular proliferation, and promoted the ability of progesterone to induce ovalbumin synthesis. However, at the doses used in this study, genistein did not induce significant growth of the oviduct as did DES, nor did it significantly increase plasma vitellogenin, which is a common marker for estrogenic exposure in a number of species.

The relatively weak effects of oral genistein as compared to DES could be due to a number of factors. Genistein is poorly absorbed from the intestine of most animals, is subject to bacterial conversion, and is rapidly metabolized in the hepatic circulation and conjugated to bile for excretion. Furthermore, it is possible that genistein does not possess the full range of activity of steroidal estrogens. Many estrogenic substances exhibit both estrogen agonist and antagonist activities through competition for receptors and plasma binding proteins. This may have been the case with genistein in this study. It is also possible that more pronounced estrogenic effects would have been induced with a longer duration of dosing. It is possible that the chicken reproductive system may be more responsive to genistein during a different period of development.

Injected dosing of genistein significantly oviduct weight, induced the appearance and growth of the normally vestigial right oviduct, and induced “adult” mating behavior

in the chicks. The injected dose of DES had similar but more pronounced effects, as expected. This bears out the observation that genistein has estrogenic activity in the chick, but that the estrogenic potency is less. The failure of genistein to significantly increase vitellogenesis, while DES did induce vitellogenin production, is consistent with genistein being a much weaker estrogen, but may also indicated that genistein is a selective estrogen in the chicken and does not express all the functions of a classical estrogen. Finally, these results demonstrate that oral ingestion of genistein results in much lower estrogenic effects on the chicken. The difference between the oral and injected dosing effects is likely to be due to reduced intestinal uptake, intestinal inactivation, and/or hepatic conjugation and excretion in the bile.

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APPENDICES

Appendix 1

Immunostaining of Paraffin Sections with Microwave Antigen Retrieval Protocol

Day 1

1. Deparaffinize:
   A. Place slides in glass slide holder back to back with 2 slides per slot. Fill any empty slots with blank slides. Deparaffinize sections in Hemo-D (2 x 10 minutes). Transfer to 100% EtOH (2 x5 min), and then to PBS (2 x 5 min).

2. Antigen Retrieval:
   A. Place holder in a glass slide bath containing 250 ml of working strength Antigen Retrieval Solution. Place lid loosely on bath and center it inside a microwave oven on a paper towel to absorb any liquid run over.
   B. Turn the oven on high power (500-1 000 watts) and closely watch the solution until it comes to a rapid boil, and then turn off oven. (Note: It usually takes 3-7 minutes before a boil is reached depending upon the type of oven, temperature, etc. It is important that a rapid boil is achieved for every run before proceeding to the next step.
   C. Set oven power to approximately power level 4 and heat for 10 to 15 minutes. (Note: The power setting should be adjusted so that the oven cycles on and off every 20-30 seconds and the solution boils about 5-10 seconds each cycle. This power setting should be noted and used for this step in all subsequent runs for the same antibody. Each antibody should be tested for the optimal time for this step.)
   D. Remove slide bath from microwave oven. Allow slides to cool for 20- 30 minutes at room temperature. Rinse with 3 changes of deionized water (dip at least 10 X per change).

3. Blocking:
   A. Place staining rack in bucket and block with PBS containing 1% BSA for 60 minutes at room temperature. Pour off excess liquid, dry with kimwipe around sections. Circle tissue with a PAP pen be careful not to touch any water with the pen.

4. Primary Antibody:

   A. Add diluted primary antibody (dilute in PBS containing1% BSA, irrelevant control antibody or preimmune control) in one or two drops to sections. Place slides in a closed dish containing wet paper towel, and place in refrigerator overnight at 4°C.

Day 2

5. Secondary Antibody:
    A. Place slides in slide holder and place in slide bucket. Wash five times in PBS containing 1% BSA over 40 minutes with gentle shaking (changing every 8 minutes).
    B. Perform procedure in the dark! Remove slider from slide holder. Dry around sections and place in a container with a wet paper towel. Apply Biotinylated Goat Anti-Mouse and incubate for 10 minutes at room tempemperature.
    C. Place slides in slide holder and rinse 4 X in PBS (dipping at least 10 X per change). Remove slider from slide holder. Dry around sections and place in a container with a wet paper towel. Apply Streptavidin Peroxidase and incubate for 10 minutes at room temperature.
    D. Place slides in slide holder and rinse 4 X in PBS (dipping at least 10 X per change). Remove slider from slide holder. Dry around sections and place in a container with a wet paper towel. Add 2-4 drops of DAB Chromogen (carcinogenic) to 2 ml of DAB substrate, mix by swirling. Apply to tissue with a dropper and incubate for 5 – 15 minutes, depending on desired stain intensity. Wash with distilled water into a closed container using a squirt bottle.

6. Dehydrate Slides:
    A. Place slides in slide holder. Place slides in 80%, 90% and 2x 100% EtOH (1 minutes each), then immerse in 2 changes of Hemo-D (1 minute each). Leave the slides in Hemo-D until the cover slips can be applied so that they do not dry out. Mount cover slips on the slides using Permount (Fisher) and examine in the light microscope.

Solutions:

Phosphate Buffered Saline (PBS)
PBS with 1% Bovine Serum Albumen (BSA)
Antigen Retrieval Buffer (ARB)   10 mM Sodium Citrate, pH 6

Calculations:

Percent Solution = g / 100 ml
PBS containing 1% BSA (Sigma A- 7030)   5 g BSA / 500 ml PBS

Molar Solution = moles solute / L solution   10 mM Soduim Citrate (F.W. 294.1)
1 M = 294.1 g / 1000 ml   10 mM = 2.94 g / 1000 ml ddH_2O or
1.47 g / 500 ml ddH_2O

H_2O: 1 ml = 1 g   100 µl = 100 mg

Primary antibody: 1:250 = 8 µl / 2 ml   1:500 = 4 µl / 2 ml

Appendix 2

Bio-Rad Protein Assay Protocol

1) Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionized water. Filter through Whatman #1 filter (or equivalent) to remove particulates. This diluted reagent may be used for approximately 2 weeks when kept at room temperature.

2) Prepare three to five dilutions of a protein standard, which is representative of the protein solutions to be tested. The linear range of the assay for BSA is 0.2 to 0.9 mg/ml, whereas with IgG the linear range is 0.2 to 1.5 mg/ml.

3) Pipet 50 µl of each standard and sample solution into a clean, dry test tube. Protein solutions are normally assayed in duplicate or triplicate.

4) Add 2.5 ml of diluted dye reagent to each tube and vortex.

5) Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.

6) Measure absorbance at 595 nm.

Appendix 3

Direct Elisa Protocol

Day 1

1. Coating the Plate:
    A. Make 1-10 µg/ml solution of antigen (peptide or protein) in PBS. Coat High Binding ELISA Strip Plates by dispensing 200 µl of antigen solution per well. Cover the plates with Adhesive Films.
    B. Incubate at room temperature for 5 hours with gentle shaking or overnight at 4ºC with gentle orbital shaking.

2. Wash:
    A. Shake off excess antigen coating solution. Wash plates 2 times with 300 µl PBST.

3. Blocking:
    A. Block remaining binding sites in each well by adding 250 µl of BSA to each well. Incubate plates overnight at 4°C with gentle mixing.

Day 2

4. Primary Antibody Solution
    A. Dilute primary antibody in BSA to a final concentration of 2 µg/ml (1:2000 dilution of stock).
    B. Add 200 µl primary antibody to each well, cover, shake and incubate for 2 hour 30 minutes at room temperature.
    C. Aspirate solution and wash plates 5 times with PBST. (300 µl wash buffer should be used for each washing; an improper washing will lead to high background).

5. Secondary Antibody Solution
    A. Add 50 µl secondary antibody (vecostain ABC ElitePK-6102 Mouse IgG kit) and 150 µl of BSA to each well.
    B. Cover, shake, and incubate at room temperature for 2 hours.
    C. Remove secondary antibody and wash 8 times with 300 µl PBST. Soak for 15 minutes between 5th and 6th washes.
    D. Refridgerate plate over lunch.
    E. ABC Reagent Prep: 2 drops reagent A to 5 ml PBS 0.1% TWEEN-20. 2 drops reagent B and mix. Stand at room temperature for 30 minutes.
    F. Add 200 µl per well ABC reagent. Cover, and incubate 30 minutes at room temperature no shaking.
    G. Remove ABC reagent and rinse 5 times with 300 µl PBST.

H. ABTS Substrate Prep: 2 drops buffer to 5 ml dH_2O in the dark and mix. Add 2 drops ABTS stock and 2 drops hydrogen peroxide from kit.
I. Add 200 µl ABTS substrate. Cover, and incubate at room temperature for 20 minutes in the dark, no shaking.
J. Read absorbance at 405.

Solutions:

Phosphate Buffered Saline (PBS)
PBS with 1% Bovine Serum Albumen (BSA)
10 mM PBS, pH 7.4 with TWEEN 20 (PBST)

Calculations:

Percent Solution = g / 100 ml
PBS containing 1% BSA (Sigma A- 7030)
  5 g BSA / 500 ml PBS
10 mM PBS, pH 7.4 with TWEEN 20 (PBST)
  0.5 ml TWEEN-20 / 1 L PBS

Appendix 4

Sandwich Elisa Protocol

Day 1:

1. Coat the Plate with Capture Primary Antibody Solution:
   A. Coat the plate with 200 µl of the capture primary antibody (rabbit anti-sea bream vitellogenin) diluted 1:2000 in PBS then cover.
   B. Incubate over night at 4ºC with gentle orbital shaking.

Day 2:

2. Wash:
   A. Remove the capture primary antibody.
   B. Wash 3 times with 300 µl of PBS-T.

3. Blocking:
   A. Block the plate by adding 300 µl blocking buffer (PBS-BSA) and cover.
   B. Incubate over night at 4ºC with gentle orbital shaking.

Day 3

4. Antigen Solution:
   A. Remove blocking buffer.
   B. Add 200 µl of antigen solution per well and cover.
   C. Incubate for 2 hours at room temperature with gentle orbital shaking.
   D. Remove antigen solution and wash 2 times with 300 ul PBS-T.

5. Detection Primary Antibody Solution:
   A. Add 200 ul detection primary antibody (mouse anti-bird vitellogenin) diluted 1:1000 in blocking buffer.
   B. Incubate for 2 hours at room temperature with gentle orbital shaking.
   C. Remove detection primary antibody and wash 5 times with 300 µl PBS-T.

6. Secondary Antibody Solution:
   H. Add 50 µl secondary antibody (vecostain ABC ElitePK-6102 Mouse IgG kit) and 150 µl of BSA to each well.
   I. Cover, shake, and incubate at room temperature for 2 hours.
   J. Remove secondary antibody and wash 8 times with 300 µl PBST. Soak for 15 minutes between 5th and 6th washes.
   K. ABC Reagent Prep: 2 drops reagent A to 5 ml PBS 0.1% TWEEN-20, 2 drops reagent B and mix. Stand at room temperature for 30 minutes.
   L. Add 200 µl per well ABC reagent. Cover and incubate 30 minutes at room temperature no shaking.

M. Remove ABC reagent and rinse 5X with 300 µl PBST.
N. ABTS Substrate Prep: 2 drops buffer to 5 ml dH_2O in the dark and mix. Add 2 drops ABTS stock and 2 drops hydrogen peroxide from kit.
O. Add 200 µl ABTS substrate. Cover and incubate at room temperature for 20 minutes in the dark, no shaking.
P. Read absorbance at 405 nm.

Solutions:

Phosphate Buffered Saline (PBS)
PBS with 1% Bovine Serum Albumen (BSA)
10 mM PBS, pH 7.4 with TWEEN 20 (PBST)

Calculations:

Percent Solution = g / 100 ml

PBS containing 1% BSA (Sigma A- 7030)	5 g BSA / 500 ml PBS
10 mM PBS, pH 7.4 with TWEEN 20 (PBST)	0.5 ml TWEEN-20 / 1 L PBS

Appendix 5

Protocol for Determining Total Isoflavones in Plasma with HPLC

Day 1

A. Freshly mix up the β-glucuronidase/sulfatase mixture by adding 0.15 g ascorbic acid, and 500 µl of β-glucuronidase/sulfatase from *Helix pomatia* to 10 ml of 0.2 *M* acetate buffer, pH 4.0.
B. Transfer aliquots of 250 µl of plasma to a 10 ml glass disposable centrifuge tube.
C. Treat with a 0.5 ml of the β-glucuronidase/sulfatase mixture to hydrolyze glucuronide and sulfate conjugates of genistein, daidzein, and glycitein.
D. Add 0.75 ml of 0.2 *M* ammonium acetate buffer to completely hydrolyze the plasma samples.
E. Cap the tubes and heat overnight (15-18 hours) at 37°C.

Day 2

A. Remove tubes from heat and cool to room temperature.
B. Add 200 µl of a 50 µg/ml solution of 4-hydroxybenzophenone in methyl tert.-butyl ether (internal standard solution) to each tube.
C. Extract the plasma sample by adding 6 ml of methyl tert.-butyl ether.
D. Mix on an end-over-end mixer for 30 minutes.
E. Centrifuge at 2000 G for 10 minutes. Transfer the ether layer to a siliconized glass culture tube.
F. Concentrate to dryness at 45-50°C under nitrogen.
G. Dissolve the residue in an appropriate amount (250 to 4000µl) of methanol-0.05 *M* ammonium formate, pH 4.0 (20:80, v/v).
H. Mix on a vortex mixer as needed for reconstitution.
I. Transfer at least 250 µl of reconstituted material to an appropriate auto-sampler vial for HPLC analysis.
J. The initial HPLC conditions were used to concentrate the analytes on the head of the column.
K. After a brief period under these conditions, a rapid gradient was used to increase the elution strength of the mobile phase. This allows some matrix components to elute, while retaining the analytes on the column.
L. After the rapid gradient, use a slower gradient to allow the desired separation to occur.
M. After the analytes elute, increase the elution strength again to elute all remaining matrix components.
N. Maintain the flow-rate at 2 ml/min in order to speed analysis time and column reequilibration. Maintain the column at 40°C.
O. Detect the analytes by UV absorption at 259 nm.

HPLC Gradient

- Start at 100% A.
- Change to (linear gradient) 40% B over 0.5 minutes.
- Hold at (isocratic gradient) 40% B for 11 minutes.
- Change to (linear gradient) 80% B over 1 minute.
- Hold at (isocratic gradient) 80% B for 3 minutes.

Solutions:

- 0.5 ml β-glucuronidase/sulfatase mixture
  - 0.15 g ascorbic acid
  - 500 µl β-glucuronidase/sulfatase from *Helix pomatia*
  - 10 ml of 0.2 *M* acetate buffer, pH 4.0
    - acetic acid is 87X, dilute 1 ml acetic acid into 86 ml HPLC water
    - acetic acid is 87X, dilute 0.25 ml acetic acid into 21.5 ml HPLC water
- 0.75 ml of 0.2 *M* ammonium acetate buffer
  - 15.416 g ammonium acetate into 1 L HPLC water
  - 0.1542 g ammonium acetate into 10 ml HPLC water
- 200 µl of a 50 µg/ml solution of 4-hydroxybenzophenone in methyl tert.-butyl ether
  - 50 µg 4-hydroxybenzophenone into 1 ml methyl tert.-butyl ether
- 250 – 4000 µl methanol: 0.05 M ammonium formate, pH 4.0
  - 3.153 g ammonium formate into 1 L HPLC water
  - 1.577 g ammonium formate into 500 ml HPLC water
    - Mix the 0.05 ammonium formate solution, pH 4.0 with methanol in a 80:20 ratio of ammonium formate to methanol.
- Mobile phase A
  - 0.05 M ammonium formate, pH 4.0
    - 3.153 g ammonium formate into 1 L HPLC water
- Mobile phase B
  - 50/50 mix of HPLC-grade methanol and HPLC-grade acetonitrile
    - 500 ml HPLC-grade methanol and 500 ml HPLC-grade acetonitrile