Phytoestrogens in Livestock

Michelle Mostrom, DVM, MS, PhD, Diplomate ABVT, ABT
NDSU-VDL, Veterinary Toxicology
Fargo, ND
Michelle.Mostrom@ndsu.edu

Phytoestrogens

Plant derived estrogens – why do they occur?
✓ Attraction of pollinators and seed dispersion,
✓ Role in disease & defense in plants.

Considered endocrine disrupting compounds (ECDs) or estrogen active compounds (EACs) with pro’s and con’s regarding occurrence in the diets and effects in animals and humans.

17ß estradiol

<table>
<thead>
<tr>
<th>Isoflavonoids:</th>
<th>Formononetin</th>
<th>Clovers (red &amp; white and others)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biochanin A</td>
<td>Soybeans</td>
</tr>
<tr>
<td></td>
<td>Daidzein</td>
<td>Beans</td>
</tr>
<tr>
<td></td>
<td>Genistein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glyceitein</td>
<td></td>
</tr>
<tr>
<td>Flavanoids:</td>
<td>8-prenylhoringenin</td>
<td>Hops (Humulus lupulus) and beer</td>
</tr>
<tr>
<td></td>
<td>Procyanidins</td>
<td>Cocoa beans, kale, citrus fruits</td>
</tr>
<tr>
<td>Stilbenes:</td>
<td>Trans-resveratrol</td>
<td>Red wine (grape skin) Peanuts</td>
</tr>
<tr>
<td>Lignans:</td>
<td>Secoisolariciresinol (enterodiol)</td>
<td>Flaxseed Tomato Squash Tea Strawberries Cranberries</td>
</tr>
<tr>
<td></td>
<td>Matairesinol (enterolactone)</td>
<td></td>
</tr>
<tr>
<td>Coumestans:</td>
<td>Coumestrol</td>
<td>Legumes (alfalfa, clover) Spinach</td>
</tr>
</tbody>
</table>

{1}
Phytoestrogens can be beneficial, benign, or harmful

**Nutraceutical use has focused on health:**
- Antineoplastic,
- Antioxidant,
- Anti-inflammatory,
- Cardiovascular, (lipoprotein profile)
- Osteoporosis,
- Probiotics.

**Veterinary focus:**
- Reproduction (infertility, early embryonic death, ovarian dysfunction, prolonged gestation)
- Prolapsed vagina, cervix, rectum,
- Mammary gland development/swelling,
- Immune changes,
- Anabolic effects.

Mechanisms of action and diversity of effects following exposure to estrogen active chemicals (EACs) that interact with an estrogen receptor are poorly understood.

The basic characteristics of EACs including the pharmacokinetics (what the body does to the drug [PK]) and pharmacodynamics (what the drug does to the body [PD]) and health outcomes are complex and not well identified.

Cellular signaling of estrogens is mediated in part through estrogen receptors – ERα and ERβ, which have large receptor ligand binding cavities allowing many different molecules to bind to and activate the ER (promiscuous binding).


**Estrogens**

Estrogens (phytoestrogens) bind to the ligand-binding domain of inducible nuclear transcription factors, ERα and ERβ, leading to activation or repression of target genes.

Antagonistic effects occur when a compound binds to receptor, but configuration can not activate the estrogen responsive genes.

Antagonistic and agonistic effects caused by phytoestrogens are responsible for the different effects as compared to estradiol.
**Estrogen Receptors**

<table>
<thead>
<tr>
<th>ERs and ERβ function in</th>
<th>Normal ovarian follicular development</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vascular endothelial cells</td>
</tr>
<tr>
<td></td>
<td>Myocardial cells</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td></td>
<td>Breast tissue</td>
</tr>
</tbody>
</table>

| ERα associated with     | Bone maturation in males and females  |
|                        | More important maintaining FSH and LH in blood |

| ERβ associated with     | Bone maintenance in females           |
|                        | Frontal lobe mediated memory and learning |
|                        | Coumestrol and genistein bind with higher estrogenic potential |

Equol modest affinity for binding (little or no ERα binding).

**Estrogen Receptors – Mechanism of Action**

ERs can be located in the nucleus as ligand-regulated transcription factors and associated with specific DNA sites and recruitment of cell-specific transcriptional coregulatory proteins, as coactivators (for gene induction) or corepressors (for gene silencing).

When ERα and ERβ are co-expressed in cells, ERβ can antagonize or inhibit ERα dependent transcription.

ERs can be located:
- in the cytoplasm, independent of gene transcription (e.g. activation of kinases, ion channels, phosphatases)
- on cell membranes

Ligand-independent pathways: for example, growth factor signaling may phosphorylate and activate ER in the absence of a ligand.

**Isoflavones**

- > 5000 identified in plants.
- Polyphenols (usually attached to sugars) providing pigmentation and flavor to plants (fruits and vegetables).
- Basic structure similar to endogenous and therapeutic estrogens.
- Health effects related to biological activity as aglycones (no sugar attachment).
- Women metabolize isoflavones more efficiently than men.
- Only 30 to 40% of humans can metabolize daidzein to the more estrogenic metabolite S-equol.
Isoflavone Structure

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Trivial Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>7,4'-OH₂</td>
<td>Daidzein</td>
</tr>
<tr>
<td>5,7,4'-OH₂</td>
<td>Genistein</td>
</tr>
<tr>
<td>7-OH, 4'-OCH₃</td>
<td>Formononetin</td>
</tr>
<tr>
<td>5,7-(OH)₂, 4'-OCH₃</td>
<td>Biochanin A</td>
</tr>
<tr>
<td>7,4'-OCH₂</td>
<td>Glycitein</td>
</tr>
</tbody>
</table>

Isoflavone Metabolism

- Most isoflavones are present as glycosides (e.g. daidzin, genistin) in plants, which are hydrolized by intestinal glucosides to release the aglycones.
- Aglycones can be metabolized in the GIT by microbes into hormone-like compounds and a portion absorbed into the blood.
- They can be conjugated by glucuronide and/or sulfate, excreted into urine, milk, and bile and can undergo enterohepatic recirculation.
- Typically 90% of circulating isoflavones and metabolites are conjugated in bloodstream.

Isoflavone Metabolism - Diagram
Pharmacokinetics: Estrogens

- Soy isoflavones, daidzein, genistein, and the estrogenic metabolite, S-equol, are very similar to estrogens allowing them to bind to ERs exerting either an estrogenic or anti-estrogenic activity.
- After ingestion and hydrolysis to aglycones in the GIT, daidzein can be metabolized to S-equol and a non-estrogenic metabolite (0-desmethylangolensin) and genistein can be metabolized to the non-estogenic p-ethyl phenol (?).
- In humans, most daidzein and genistein are conjugated in plasma, with < 1.5% of the total plasma pool of genistein unconjugated.
- In humans, about 50% of absorbed equol appears in free form.
- Response to estrogenic chemicals is dependent on the status of the tissue at the time of estrogenic exposure (i.e., responses are specific to the hormonal history of the tissue).
Modulation of Aromatase by Phytoestrogens

The aromatase enzyme catalyzes the conversion of androgens (androstenedione and testosterone) to estrogens in many tissues. Phytoestrogens (particularly biochanin A and formononetin) act as aromatase inhibitors:

- ↓ aromatase gene expression (CYP19 in human tissues)
- Inhibit the aromatase enzyme itself.

Isoflavone Metabolism by Microbes

Formononetin → Daidzein → Equol

Equol is a more estrogenic compound than formononetin and can act as an antiestrogen.

Equol can exist as S- or R- forms; S-equol is the natural chemical and a more potent estrogen, and shows greater higher binding affinity towards ERβ than ERα.

Cattle have about 10X free equol as compared with sheep and excrete more equol in urine.

Isoflavones in Plants

- The main soya isoflavones are genistein, daidzein, and the estrogenic metabolite S-equol.
- The predominant isoflavones in red clover (*Trifolium pratense*) are biochanin A and formononetin, respectively the methylated derivatives of genistein and daidzein. Formononetin can be metabolized to S-equol.
- Red clover can have 30X isoflavone content of soybeans.
- Isoflavone types and concentrations can vary between red clover harvested at the early bud stage and late flowering stage, with higher isoflavone concentrations post-flowering.
- Higher isoflavone concentrations found in the LEAF > STEM > PETIOLE > FLOWER (some studies suggest flowers are highest in isoflavones).
- Red clover silage has been reported to have 18% higher isoflavone concentrations than non ensiled, wilted herbage.
Red Clover Cultivars

- Multi-year, multi-site trial in Quebec found total isoflavone concentrations in 10 cultivars ranged between 8923 and 12753 µg/g of DM.
- One cultivar, "Start", consistently had low isoflavone concentrations for formononetin and biochanin A.
- Strong environmental effects – such as soil types and soil fertility, soil moisture, prevailing environmental conditions.
- Isoflavone concentrations were highest in the leaves > stems > flowers. Some changes with plant maturity.
- Harvest red clover as a forage source for ruminants at early flowering stage to minimize isoflavone concentrations.
- Isoflavone content 22% higher in fresh material than either silage or hay. Feeding red clover hay, rather than fresh herbage, could reduce adverse effects because of a lower formononetin content.

Equol

- Equol was first isolated from equine urine in 1932 and wasn't identified as a isoflavone metabolite produced by intestinal bacteria until 50 years later.
- Non-steroidal estrogen produced by specific bacteria in the GIT on animals or humans eating daidzin or daidzein (formononetin).
- All animal species tested produce equol following isoflavone ingestion.
- 50 to 60% of Asian adults are equol producers compared to 25 to 30% of Western adults.
- Selective ERβ agonist with weak activity for ERα.
- Species differences in equol metabolism.
- Antagonistic to estrogen through competitive binding to cytoplasmic ERs.
- Potent antagonist to dihydrotestosterone.

8-prenylnaringenin (8-PN) in Hops

- 8-PN is found in hops (Humulus lupulus) and in beer, and is responsible for some of the estrogenic effects of hops.
- 8-PN can bind to both α and β estrogen receptors and can up-regulate the progesterone receptor mRNA using in vitro cell lines.
A farmer in the north of France, having been driven by the scarcity of fodder to try to make use of whatever fell in his way for feeding his cattle, prove that hop leaves were a valuable element of food for cows when mixed with other substances. He found that whenever he gave them hop leaves he always obtained more milk and his cows thrived better than usual. The leaves must be used as soon as they are plucked, for the cows object to them when dried by the sun.

---

**Metabolic Transformation of Lignans**

<table>
<thead>
<tr>
<th>Glycosides</th>
<th>Hydrolyzed</th>
<th>Aglycones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matairesinol</td>
<td>Rumen microbes</td>
<td>Enterodiol</td>
</tr>
<tr>
<td>Enterolactone</td>
<td>Colon microbes</td>
<td>(absorbed, conjugated, undergo enterohepatic recycling, found in milk, urine, feces).</td>
</tr>
</tbody>
</table>

---

**Dairy Cow Metabolism of Phytoestrogens**

Rumen cannulated lactating Norwegian Red cows fed baled silage of: 1) red clover-timothy, 2) native grassland forage, 3) perennial ryegrass, 4) timothy (n=1 cow/trt, silage fed at 70% total dry matter intake for 3 weeks).

Isoflavones were extensively metabolized in the rumen (85-99%), with some lower intestinal metabolism.

Plant (e.g., matairesinol & secoisolariciresinol, etc.) lignans degraded in the rumen; primarily excreted in feces.

Main phytoestrogen excretion through feces and urine, with only a small portion excreted in milk.

Phytoestrogen content in milk is important in EU.

Isoflavone Metabolism

- Rumen microbes adapt to phytoestrogens in the ration and will become more efficient in metabolism over time.
- Biochanin A and genistein will be almost completely metabolized to non-estrogenic compounds over time (~ 10 to 14 days).
- From *in vitro* work, formononetin and daidzein have longer half-lives in rumen.
- Less equol is absorbed from the rumen of cattle than of sheep and, therefore, more equol is excreted in cattle feces and sheep urine.

Alfalfa (*Medicago sativa*) – Effects of Coumestrol

Several references suggest that alfalfa containing 25 to 30 mg/kg (ppm) coumestrol fed to cattle can cause hyperestrogenism; however, the inclusion rate / percentage of dry matter is not mentioned.

Dairy cattle fed alfalfa (implied high coumestrol concentrations) can develop cystic ovaries and irregular estrous cycles, precocious mammary and genital development in heifers.

Coumestans can suppress estrus and inhibit ovulation.

Coumestrol Effects

Coumestrol has estrogenic and anti-estrogenic effects. It has been shown in lab animal studies to:
- ↓ frequency of ovulation,
- ↑ risk of ovarian cysts and estrous cycle duration,
- ↑ embryo mortality,
- ↓ aromatase activity and blood estradiol concentrations.

A study using bovine luteal cells from 3rd to 5th, 6th to 8th, and 9th to 12th week of pregnancy were incubated with coumestrol for 48 hrs. Findings were:
- No change in progesterone (P4) secretion,
- ↑ oxytocin (OT) secretion at all stages, hence ratio of P4/OT ↓,
- ↑ expression of neurophysin-I/oxytoxin/OT mRNA (oxytocin prohormone),
- ↑ peptidyl-glycine-α-amidating mono-oxygenase (post-translational oxytocin synthesis),
- ↓ Prostaglandin E2 secretion at all stages.

Coumestrol impairs secretory function of CL and maintenance of pregnancy.

Legumes and Fertility Review – Alfalfa/Lucerne

- Coumestrol is the primary phytoestrogen, with the higher concentration found at the budding stage of growth.
- Stress factors, such as pests, diseases, and environment can increase coumestrol production.
- Research into effects in ewes consuming coumestrol in alfalfa showed minimal effects or increased cervical folds and smaller ovaries, with no difference in ovarian activity (small research study).
- Main bovine research from Romero et al. (1997) using alfalfa silage (66.8 mg/kg coumestrol) fed to dairy cows for one year:
  - 608 cows
  - 378 pregnancies (36 aborted)
  - 1264 inseminations
  - 102 cows had increased cervical mucous and turgid uterus at the palpation of a positive diagnosis of gestation between 40 to 60 days.
  - Only 238 normal pregnancies.
  - Syndrome observed in the cows: repeat breeding, abortions, metritis, ovarian cysts, and other estrogenic manifestations.


Ranking of Estrogenic Potency of Phytoestrogens for Both ER subtypes

ERα: Estradiol >> zearalenone = coumestrol > genistein > daidzein > apigenin = phloretin > biochanin A = kaempferol = narigenin > formononetin = ipriflavone = quercetin.

ERβ: Estradiol >> coumestrol = genistein > zearalenone > daidzein > biochanin A = apigenin = kaempferol = naringenine > phloretin = quercetin = ipriflavone = formononetin.

Sources & Estrogenic potency of phytoestrogens: Livestock

Isoflavones:
Soybeans and grassland legumes, e.g. red and white clover

Lignans:
Primarily found in cell walls of seeds from grain, legumes, and linseed used in concentrates.

Coumestans:
Coumestrol is the major compound found in legume seeds and Lucerne (Medicago sativa) and white clover.

Based on in vitro and in vivo studies:
Estradiol > coumestrol > equol and genistein > glycitein > daidzein > formononetin, biochanin A
In 1946 in SW Australia, a massive problem of 'failure to breed' with 30% fertility, cystic glandular hyperplasia of the cervix and uterus, and lactation in nonpregnant ewes and wethers observed in sheep grazing subterranean clover.

- The isoflavones identified in the clover were genistein and formononetin.
- The estrogenic activity of sub clover pastures was correlated with formononetin concentrations, which has low estrogenic activity.
- The discovery of formononetin metabolism to daidzein and bioactivation to equol by rumen microbes explained the effects.
- After sheep grazed sub clover pasture for several years, the flock fertility declined to zero with permanent infertility.
- The cervix and vagina of ewes failed to respond normally to endogenous estrogen to 'prime' the cervix, and cervical mucous had an altered consistency resulting in poor sperm penetration to the oviduct with altered cervical tissue morphology.
- Often no visual signs that ewes are infertile as ovarian function remains normal and normal estrous cycles.
- Reduced period of CL function and changes in progesterone may explain some of the ewe infertility.

**Legumes and Fertility Review – Red Clover**

Usually high phytoestrogen content in the spring with first cuttings, and declining phytoestrogens after flowering.

Nutrient deficiencies that impair red clover plant growth can increase formononetin concentrations, and hay and silages can retain phytoestrogen content.

Silage with 0.56% formononetin (5,600 mg/kg) in the dry matter of spring red clover caused fertility disturbances in dairy cows. [I would be concerned at 1/5 of that number.]

Findings from several studies: ensiled red clover could still lead to reduced ewe fertility when offered as ≥ 25% of the diet.


**Legumes and Fertility Review – White Clover**

- White clover (*Trifolium repens*) is a widely grown protein source, usually in a mixed grass / clover pasture. Can produce isoflavones and coumestrol when under stress.
- Most common factor stimulated coumestrol production is fungal diseases of leaves, however insect damage, virus infection, and poor plant nutrition can increase coumestrol.
- Coumestrol levels in white clover DO NOT vary with stage of growth.
- Main effects are reduced ovulation and delayed estrus in ewes, and reduced conception rates and milk-like secretion in heifers reported in bovine.
Melilotus officinalis
M. alba

Feeding sweet clover hay from a pasture invaded by these plants, haying was delayed in the fall and plants were quite mature. No dicoumarol detected.

Significant amounts of coumestrol in various cuttings: 35 ppm, 12.5 ppm, 7.5 ppm.
Low formononetin levels: 14.5 ppm and 24 ppm (insignificant but additive in effect)

Dairy Cattle in the Winter ’08 – ’09

• Feeding 20 lb haylage and some corn to heifers, with a poor conception rate.
• Feeding 11 lb haylage to cows, with some return to service, lack of dominant follicles on ovary.
• Feeder steers bulling; freemartins in heat.
• Cows towards the end of lactation stop milking.
• Analysis of haylage found 70 ppm coumestrol and 23 ppm formononetin.

Dairy Cows & Phytoestrogens

In high producing dairy cows pushed on forage/haylage, NDSU cases suggest that > 18-20 ppm coumestrol in forage is problematic and associated with early, embryonic death and return to service.
Daidzein Enhances Cow Immune Function

- Holstein cow is sensitive to heat stress when the temperature is > 77 °F (25 °C) and the temperature humidity index (THI) > 72, with thermal neutral at 20 °C and 20% humidity and THI of 64.
- This study evaluated daidzein to enhance immunity (serum immunoglobulin G, interferon-α, interleukin-2) in late lactation cows under heat stress.
- Cows (n=10) fed daidzein at 0, 200 mg/day, 300 mg/day, and 400 mg/day added to the ration for 60 days (with no measurement of baseline isoflavone concentration in the ration with alfalfa, pea shell, soybean meal).
- Average weather: 33.5 °C, 62% humidity, and 84.5 THI.

Higher daidzein supplementation at 300 and 400 mg/day increased serum IgG, IFN-α, and IL-2.


Potential Risk of Isoflavones: Piglets

- 23 day old weaned crossbred pigs were supplemented in the feed (no soy source) with daidzein at control, +40 mg/kg, +200 mg/kg, and +400 mg/kg for 70 days.
- 400 mg daidzein/kg feed resulted in lower average daily weight gain (0.47 kg vs. 0.54 kg) and some histological changes in the spleen.
- Results suggest that daidzein dietary supplement of 400 mg/kg negatively affects weight gain and splenic morphology of young pigs.


Late Winter ‘08

History provided with the case that the palpation mares at a Mid-west Veterinary College were all lactating, even those not pregnant.

Ration:
- Alfalfa hay
- Equine maintenance pellets
- Equine senior diet
Summer - Fall ‘08

Dressage stable:
- Early embryonic loss in mares
- Unusual follicular dynamics in 4 mares

Clover pasture – contained isoflavone phytoestrogens:
- 910 ppm formononetin
- 595 ppm Biochanin A

Interesting comment: laminitis is a common problem in horses grazing grass and clover (Cheek & Shull, 1985, Natural Toxicants In Feeds and Poisonous Plants).

Coumestrol Exposure in Mares

- Mares were individually housed in stables and fed alfalfa and clover haylage, concentrate meal and hay pasture for 4 to 9 months (October – July). Haylage coumestrol was 3.1 mg/kg and methoxycoumestrol was 10.4 mg/kg.
- All mares showed uterine edema while fed haylage.
- During breeding season, no ovulation occurred despite follicular growth or hCG administration. The anovulatory follicles reached 50 mm in diameter, but did not rupture. Dominant follicles persisted for >7 days and then underwent luteinization. Excessive cervical mucous discharge noted. The blood estrogen concentrations were high and progesterone levels were low.
- After haylage withdrawal, the uterus returned to normal and normal ovarian cycling occurred within 2 to 3 weeks.

Phytoestrogens

Animals have evolved with plants, particularly some lab animals on soy diets.

Phytoestrogens vary with:
• Climate (cool, wet spring/fall increase concentration),
• Plant pathogens,
• Plant maturity (higher levels the years after seeding),
• Plant part (generally leaves > stems > flowers),
• Processing: (concentrations higher in fresh > silage > hay)
  Isoflavones ↓ with delayed harvest and wilting in swaths
  Coumestrol ↑ with wilting in swaths
• Cultivars have little impact in alfalfa.

Isoflavones Affect Estrogen Action

✓ Bind ERs:
At low concentrations genistein has agonist binding activity for both α and β ER, with more affinity for ERβ. For example: hepatocytes do not express ERβ which explains why soy isoﬂavones do not affect serum lipid proﬁles or induce the prothrombotic effects associated with increased risk for thromboembolic disorders. Whereas vascular endothelium express both α and β ER which explains the positive effects of soy isoﬂavones on endothelial function in postmenopausal women. Osteoblasts express ERβ which may mediate the beneﬁcial effect on soy isoﬂavones on bone metabolism and ERβ appears to play an anti-proliferative role in healthy prostates.

✓ Influence sex steroid action by inhibitory effects on many enzymes involved in steroid metabolism. Isoflavones can down-regulate aromatase mRNA in human granulosa-luteal cells which may affect in situ steroidogenesis.

✓ Induce non-genomic actions that include effects on plasma membranes and cell signaling pathways.

Phytoestrogens’ Effect on Pituitary?

• Genistein and coumestrol compete with 17β-estradiol for binding site in hypothalamic and pituitary tissue of sheep.
• In vitro study with ovine pituitary cells (young castrated male) and 48 hour incubation with estradiol and phytoestrogens.
• Coumestrol, genistein, and zearalenone signiﬁcantly decreased basal secretion of FSH (which stimulates ovarian follicular growth and maturation) and reduced total FSH production – estrogenic response.
• Coumestrol and zearalenone enhanced secretory LH response by a GnRH agonist (D-Ala6,des-Gly10 GnRH ethylamide) – a measure of gonadotrope responsiveness to estrogen.
• Both results are dependent on an estrogen receptor.

Effects on Males

- Most limited research into effects of phytoestrogens on males does not indicate negative effects on puberty, sperm numbers (motility, morphology), reproductive traits.
- Often report anabolic effect in males at diet phytoestrogen levels causing reproductive problems in females.

A review of forage phytoestrogens in livestock – Thank you.

Questions?

"Listen Buddy! After the hot flushes I’ve been having this is like a resort!"