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Dietary red clover (*Trifolium pratense*) induces oviduct growth and decreases ovary and testes growth in Japanese quail chicks

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Abstract

To determine whether drought-stress alters phytoestrogens in red clover and whether red clover in the diet influences sexual development in Japanese quail, we fed chicks diets containing irrigated or non-irrigated clover. Irrigation altered phytoestrogenic activity of red clover (determined using an *in vitro* bioassay), with extracts of irrigated clover diet containing more estrogenic activity than extracts of non-irrigated clover diet. Chick growth was negatively correlated with the amount of irrigated or non-irrigated clover in the diet. Dietary red clover also depressed both absolute and relative gonad weights; however, relative oviduct weight was increased by the irrigated diet. Diets did not affect serum vitellogenin. These results reveal a negative influence of drought-stress on phytoestrogenic potency of clover, and that red clover in the diet can inhibit avian growth and development independent of irrigation state. Thus, phytoestrogens may affect reproductive development in wild birds, and environmental stressors may influence levels of phytoestrogens in the field.

Keywords

Phytoestrogens; Red clover; Avian; Reproduction; Endocrine disruption; Environmental estrogen

1. Introduction

Endocrine disrupting chemicals are exogenous chemicals that disrupt the natural hormone functions of vertebrates, invertebrates, and even plants [1–3]. Environmental estrogens are chemicals that either mimic or block natural endogenous estrogens, and include many man-made chemicals (termed xenoestrogens), including pesticides, industrial chemicals, plasticizers, and synthetic hormonal birth control [4]. Many of these chemicals have been

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shown to have adverse reproductive and physiological effects on wildlife [4] and possibly humans [5]. Less is understood, however, about the role of natural environmental estrogens (i.e. plant-produced phytoestrogens). Phytoestrogens are a subset of plant secondary compounds (SPCs, so-called because they have no function in the primary roles of growth and development in plants), and include several classes of chemicals [6]. While phytoestrogens have been shown to exert endocrine disrupting effects on many species of vertebrates, including livestock [7,8], rodents [9–11], fish [12], and birds [13,14], little is known about the evolutionary function of these chemicals in plants. It has been suggested, however, that phytoestrogens have evolved as a chemical defense against vertebrate herbivory [15–17], similar to the well-studied ecdysteroidal disruption that plants exert on certain insects [18].

In birds, as in mammals, administration of estrogens to embryos disrupts normal sexual differentiation in both males and females. The effects of exogenous estrogen exposure on gonad differentiation have been well studied in galliformes, and it is well established that estrogen feminizes gonadal and sex duct development in these species [19]. Early studies indicate estrogen is required for feminization of the gonads: the removal of the left ovary from immature chickens resulted in the right gonad developing into a testis [20], and embryonic exposure of chicks to estrogen feminizes the embryonic gonad, resulting in gonads that are histologically ovarian [21]. *In vitro* studies showed similar results, with estrogen inducing cortical growth of cultured undifferentiated chick gonads [22]. Recently, studies have shown that exposure of Japanese quail embryos to low doses of ethynylestradiol (EE2) (2 ng EE2/g egg) results in right-side oviduct retention and left-side structural malformations in female Japanese quail chicks and ovary-like tissue in the left testis of male chicks [23,24]. Additionally, ovotestes were produced in male quail in response to embryonic exposure to estrogen [25], and female quail embryonically exposed to the xenoestrogen o,p'-DDT retained right oviducts and had decreased length of left oviduct [26]. Panzica et al. [27] suggested that estrogen-induced reduction of the sexually dimorphic bed nucleus of the stria terminalis provides a sensitive end-point for exposure to the de-masculinizing effects of estrogen in male chicks during the organizational period. This nucleus supports male copulatory behavior [27] and embryonic exposure of xenoestrogens to Japanese quail results in reduced male copulatory behavior [28–30].

Clearly, developmental exposure of exogenous estrogens affects adult reproduction in birds, but what role do phytoestrogens in the environment have on wild populations? In the only study of its kind, Leopold et al. (1976) sought to link phytoestrogens in the environment with the reproductive success of a population of wild California quail. They found that in years with high precipitation, there were low levels of phytoestrogens in the vegetation eaten by the quail, and there was high reproduction (325 young per 100 adults). During a year of low precipitation, there were high levels of phytoestrogens in the vegetation and the vegetation was sparse and stunted. Although there was adequate plant material for consumption, adults ate smaller amounts of food and fewer young were produced (25 young per 100 adults). The authors postulated that drought-stress induced production of phytoestrogens in the plants, which led to reproductive disruption in adult birds and fewer young produced. The authors further suggested that the high levels of phytoestrogens in the environment during stressful years are an evolutionary signal, causing quail to produce fewer young during times of low food availability [31].

While the study by Leopold and coworkers presents interesting implications regarding the role of phytoestrogens and the possible co-evolution of plants and herbivorous birds, it is purely correlative, providing no significant cause-and-effect data demonstrating that phytoestrogens in the diet disrupt reproduction. Further, Cain et al. (1987) found that only large amounts of the phytoestrogen biochanin A decreased fertility in scaled quail,

suggesting that animals would not consume enough in the field to cause reproductive disruption [32]. Red clover (*Trifolium pratense*) is commonly eaten by California quail [33], and contains the phytoestrogens formononetin and biochanin A [34]. Livestock grazing on red clover and other subterranean clovers can exhibit what is known as “clover disease” resulting in behavioral and physiological reproductive disruption in both males and females, leading to infertility [7,8,35]. Although the phytoestrogen genistein has been shown to act as an estrogen in zebra finch and chickens [13,36], few studies have considered the endocrine disruptive effects of whole estrogenic plants in birds (or, for that matter, any other vertebrate). Accordingly, here we describe studies directed toward determining whether drought-stress changes phytoestrogenic activity of red clover and whether red clover as a dietary component can influence growth and/or sexual development in Japanese quail. If phytoestrogens in the diet are induced by drought-stress and can affect reproductive endpoints in developing birds, it is then possible that phytoestrogens present in wild diets can affect the reproduction of wild birds, indicating a previously unstudied ecological relationship between birds and plants.

2. Methods

2.1. Production of red clover and diet formulation

Red clover seeds (Cal/West Seeds, Woodland, CA) were planted in October 2006 and irrigated via winter rain until harvested by cutting (close to the ground, leaving the roots) in April 2007. The total average rainfall between October and April in the Sacramento area is approximately 40 cm, with an average maximum occurring in January of 9.4 cm [37]. The first harvest (of irrigated red clover) was baled and stored. After the initial cutting, the rooted red clover was allowed to continue growing for two more months, with no additional irrigation, and harvested in June 2007. The total rainfall average between April and June is 4.1 cm [37]. Plants were then harvested and baled (non-irrigated red clover). Both irrigated and non-irrigated red clover were milled (including seeds, stems and leaves) to incorporate them into a crumble-type, formulated diet.

Irrigated red clover (IRC) and non-irrigated red clover (NIRC) were analyzed for protein and mineral content by the Division of Agricultural and Natural Resources' Analytical Laboratory, University of California, Davis. This information was used to formulate diets that contained 60% clover (either IRC or NIRC) that met the requirement for growing Japanese quail chicks as recommended by the National Research Council [38]. The remaining 40% of the diet consisted of required nutrients; the protein source was ARCON® S Soy Protein Concentrate (ADM, Decatur, IL), a soy protein with low levels of isoflavonoids. The control diet was N.A.F. 25% Turkey/Pheasant/Gamebird Starter Crumble with Amprol (Bar-Ale N.A.F., Inc., Williams, CA), which was essentially nutritionally identical to the formulated diets. As no birds showed any evidence of coccidiosis, the Amprol, a coccidiostat, was essentially an inactive ingredient. Mixtures of the red clover and control diets were made to assess a possible dose–response on the endpoints. Therefore, the diets used were: a control diet (CON); three diets containing 20%, 40%, and 60% IRC (20IRC, 40IRC, and 60IRC, respectively); and three diets containing 20%, 40%, and 60% NIRC (20NIRC, 40NIRC, 60NIRC, respectively).

2.2. Assessment of estrogenic potency of diets

All glassware used for extractions was baked for 8 h at 275 °C, and pre-rinsed with solvent. Ten grams each of the CON, 60IRC, and 60NIRC diets were extracted four times in HPLC-grade ethanol (EtOH, Sigma, St. Louis, MO) for 20 min each in a 55 °C ultrasonic water bath, with a final fifth extraction for 60 min. After each extraction, the solvent was decanted off and pooled, and fresh EtOH was added to the sample. Pooled extract was evaporated

under nitrogen gas to a volume of approximately 1 ml. Extracts were stored in amber glass vials at 4 °C. For the cell bioassay described below, samples were serially diluted in EtOH and cells were exposed to a range of approximately 10 ng–20 mg extract equivalents in solution to generate a potency curve.

Recombinant human ovarian carcinoma cells (BG-1), stably transfected with an estrogen responsive luciferase reporter plasmid (referred to as BG1Luc4E2 cells), were used for the bioassay. These cells respond to estrogenic chemicals with the induction of luciferase in a chemical-, time-, dose-, and estrogen receptor-dependent manner [39]. Initially BG1Luc4E2 cells were grown in 10 cm plates in a standard medium (α -minimum essential medium [α -MEM, Gibco, Invitrogen, Carlsbad, CA]) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Laurenceville, GA). Cells were incubated at 37 °C, 5% CO₂, and 85% humidity. Prior to treatment, cells were maintained for at least 6 days in estrogen-stripped media (ESM), which consists of α -MEM without phenol red (Sigma) containing 10% charcoal/dextran-treated FBS, changing the medium every day.

For the assay, cells were trypsinized (trypsin, Gibco, Invitrogen, Carlsbad, CA) and added to 96-well plates at a concentration of 750,000 cells per well in ESM. Cells were allowed to grow overnight, followed by removal of the media and addition of the samples and standards dissolved in media at a final solvent concentration of 0.1% (v/v) and incubated for 22 h. After incubation, media was removed, cells were washed twice with PBS, and lysed with 50 μ l lysis buffer (Promega, Madison, WI) per well and plates shaken for 20 min at room temperature. Luciferase activity in cell lysates was measured in a FLUOStar Optima, BMG Labtech with a delay time of 10 s and an integration time of 10 s after automatic addition of 50 μ l of luciferase reagent (Promega).

2.3. Animals

Day-old Japanese quail chicks were obtained from the Department of Animal Science, University of California's Avian Breeding Facility. Quail chicks were housed in 50 cm $\times$ 30 cm $\times$ 20 cm brooder pens, containing approximately 12 chicks per pen, for a total of 21 pens. Pens were stacked in racks, each rack containing 12 pens. Chicks were kept at approximately 35 °C for the first week, and 32 °C for the second week. Chicks were kept on 24 h light, and at approximately 80% relative humidity. Diets were randomly assigned to each pen; each diet was fed to the chicks of three separate pens. Food and water were provided *ad libitum*, and chicks were weighed every 2–4 days. On Day 14, blood samples from each chick were taken via wing prick (brachial vein), chicks were euthanized via sodium pentobarbital overdose, and gonads were removed and weighed. All animal care protocols were approved by the University of California's Institutional Animal Care and Use Committee.

2.4. Serum protein analysis

Protein analysis was performed using the Bio-Rad dye binding protein assay protocol. The protein dye reagent was prepared according to the Bio-Rad protocol. An aliquot of 50 μ l of each serum sample (diluted 1:50 in distilled water) was transferred to tubes, followed by the addition of 2.5 ml of the prepared dye reagent to each tube. The tubes were vortexed and transferred to plastic cuvettes and absorbency at 595 nm determined using a Spectronic 20 Genesys.

2.5. Serum vitellogenin assay (ELISA)

Multiwell (96-well) microplates (Nunc, Thermo-Fisher, Rochester, NY) were coated with the primary capture antibody solution (rabbit anti-sea bream vitellogenin, Biosense Labs, Bergen, Norway), diluted 1:1000 in PBS (Sigma, St. Louis, MO). BSA was stripped from

the antibody according to the BSA/Gelatin Removal Protocol (Pierce, Rockford, IL). An aliquot (200 µl) of the diluted antibody was added to each well. The wells on the periphery of the plates were not used in order to reduce inter-well variation. Plates were gently agitated on a shaking platform (Belly Dancer, Stovall Life Science, Greensboro, NC) overnight at 4 °C. The following day the primary capture antibody was removed and the plate was washed three times with the 300 µl 0.05% PBS-T (PBS with a 0.05% dilution of Tween-20, pH 7.4) (Sigma). Wells were then blocked with 300 µl of PBS-BSA (a 1% solution of BSA [Sigma A-7030] in PBS [Sigma]). Plates were incubated overnight at 4 °C with agitation. The following day the blocking buffer was removed from the plates, and each well was washed twice with 300 µl PBS-T (Sigma).

An aliquot (6.25 µl) of each sample was diluted in 312.5 µl of distilled water and 20 µl of each dilution was added to each well (with two replicates) and an additional 20 µl of PBS (Sigma) was then added to all wells. The mouse anti-bird vitellogenin antibody (Biosense Labs) was diluted 1:1000 in PBS-BSA (Sigma) blocking buffer and 200 µl of the antibody solution was added to each well. Plates were incubated for 2 h at room temperature with agitation, followed by washing five times with 300 µl PBS-T per well.

The secondary antibody solution was prepared according to the ABC kit instructions (Vectastain Elite ABC Kit Mouse IgGPK-6102, Vector Labs, Burlingame, CA). An aliquot (200 µl) of the secondary antibody solution was added to each well and the plates incubated for 2 h at room temperature with gentle agitation. The ABC reagent was prepared according to kit instructions and left to sit for 30 min. When the incubation period of the plates was finished, the secondary antibody was removed and wells washed eight times with PBS-T, followed by addition of 200 µl of the ABC reagent to each well and incubation for 30 min, without shaking, at room temperature. The ABC reagent was then removed and the plates were washed eight times as described as above. After washing, the ABTS substrate (Vector Labs) was prepared according to the kit instructions and 200 µl of ABTS substrate was added to each well, the plates incubated in the dark at room temperature for 20 min, followed by measurement of absorbance at 405 nm in a Biotrak II (Amersham Biosciences, Piscataway, NJ) microplate reader.

2.6. Diet potency calculations

Potency estimates for Experiment 2 were calculated using a Hill's sigmoidal 4-parameter curve fitting model in SigmaPlot (Systat, San Jose, CA):

$$y=y_0+\frac{ax^b}{c^b+x^b}$$

where y_0 is the minimum, a equals the range ($\max[y] - \min[y]$), b equals 1, and c is the transition center of the curve, i.e., the concentration that causes 50% efficiency (EC_{50}). Data points that dropped off after maximum activity was reached were not included in the curve-fit calculation, as these points indicated that the cells had sickened or died due to high concentrations of extract. EC_{50} values of the standard curve were equated, and relative luciferase units (RLUs) of the extract could be expressed in equivalents of 17- β -estradiol (E2) at the EC_{50} values. Estimated EC_{25} values were also compared. In our study, the red clover diets exhibited “super-induction”, that is, their maximum luciferase values were higher than the maximum values of the standards. In these cases, although the EC_{50} values are not within range of the standard EC_{50} values, the assumption is made that the super-induction is due to processes downstream of the estrogen response element (ERE), and the

values are still equated, normalizing the maximum value of both the standard and sample to 100% induction.

2.7. Statistical analysis—chick and gonad weights

Chick weights at Days 1, 3, 7, 11, and 15 were analyzed for significant differences using SAS, Version 9.1 (SAS Institute Inc., Cary, NC), PROC MIXED with the random variable of group, a fixed effect of diet treatment, a fixed time period (day), and interaction of time and treatment, to account for repeated measures over time and within groups. Due to the large weight differences among groups and days, the data did not meet the assumption of normality, but ANOVA was still used due to its robust nature, and the fact that repeated measures cannot be addressed in non-parametric analysis. Chick gonad weights were analyzed for significant differences using SAS, PROC MIXED with the random variable of group and a fixed effect of diet treatment to account for repeated measures within groups (the individuals in each pen were considered repeated measures). For the gonadosomatic index analyses, each individual's gonad weight was divided by the individual's bodyweight, and the resulting ratio was used for analysis, using the same SAS programming mentioned above. All data met the assumptions for normality and homogeneity of variances. Each diet had three groups (pens), and within each group there were a varying number of individuals, ranging from 7 to 13 chicks. These numbers changed somewhat over the course of the experiment due to normal levels of mortality of the chicks.

2.8. Statistical analysis—serum protein and vitellogenin levels

Ten individuals from the CON, 40IRC, 40NIRC, 60IRC and 60NIRC groups were randomly selected for vitellogenin and protein analysis. The 20IRC and 20NIRC groups were not analyzed due to limited resources. For the serum level data, all assumptions for ANOVA were met, and data was analyzed using SAS, PROC GLM, using a Tukey comparison test to assess differences among treatment groups. The vitellogenin data was analyzed by using the absorbance data for the vitellogenin level for each individual and dividing by the serum protein level (mg/ml) of the same individual, to obtain a standardized vitellogenin value. This final data set met all assumptions for ANOVA, and was analyzed in SAS, PROC GLM. For the vitellogenin analysis, all diet groups analyzed had 10 individuals, except for the 60NIRC group, which had 8 individuals, due to insufficient amount of serum for analysis.

3. Results

3.1. Estrogenic potency of diets

The red clover diet extracts had estrogenic activity ranging from approximately 7.0–49.4 pmol E2 equivalence per gram diet. The 60IRC diet had the higher activity (20.2–49.4 pmol E2 equivalence per gram diet), while the 60NIRC diet had a lower activity (7.0–10.0 pmol E2 equivalence per gram diet). The CON diet actually exhibited the highest estrogenic activity (40.0–62.5 pmol E2 equivalence per gram diet). When normalizing the BG-1 assay results, it appears that the control diet had higher estrogenic activity than the red clover diets; however, the irrigated red clover is more potent when direct comparisons of the results are compared at the EC₅₀ concentration of E2. Table 1 shows the estrogenic potencies of the diets at EC₂₅ and EC₅₀. Fig. 1A shows a sample estrogen curve, and Fig. 1B shows the absolute luciferase induction values of the three diets. The EC₅₀ values of the normalized curves are indicated by dashed lines.

3.2. Chick weights

Chicks consuming the 40IRC/NIRC and 60IRC/NIRC diets showed dramatic decreases in body mass growth over the 2-week period. On post-hatch Day 15, the CON group had

gained approximately 34.5 g. The other groups had significantly less weight gain, in a dose-response manner (with the 30IRC and 30NIRC not significantly different from each other). The most depressed growth occurred in the 60IRC diet, which gained only about 7.0 g over the course of 15 days. In general, the IRC diets depressed growth more than the NIRC diets, and on Days 11 and 15 in the 40 and 60 groups, this difference was significant (Fig. 2).

3.3. Chick gonad weights

Gonad weights are presented both as a percent of body mass (gonadosomatic index, GSI) as well as absolute weights. Increasing red clover content of the diet led to progressive declines in both absolute and relative (GSI) testes weights/ratios. All the red clover diet groups had significantly lower absolute testes weights and testes GSI than the controls, with progressively lower values as the concentration of both irrigated and non-irrigated red clover increased; no differences of irrigation status was detectable (Fig. 3A and B).

The absolute oviduct weights also decreased at the higher diet treatments. The 20IRC and 20NIRC groups had a lower weight than the CON group (although not significantly so) and all the other diets were significantly lower than the CON (Fig. 3C). The GSI for the oviducts showed a markedly different pattern of significance, with the 60IRC group showing an increased mean over all the other groups, and a significant difference from the 20NIRC groups and the 40NIRC groups. All treatments except the 60IRC groups had very similar means and were not significantly different from each other (Fig. 3D). Additionally, the 60IRC groups contained a value which was higher than all other values (18 mg absolute weight, and 0.0014% GSI), and was determined to be an outlier using the Grubbs test. This outlier was removed from analysis. Thus, in the 20% and 40% red clover diet groups there was no difference in the diet treatments, but in the 60% red clover diet groups, the irrigated clover diet significantly induced oviduct growth over the control, while the non-irrigated clover diet did not.

The absolute ovarian weights were lower in the higher doses. Both the IRC and the NIRC diets showed a dose-response, with treatment diets progressively and significantly lower than the CON diet, but the animals fed IRC diets appeared to be more strongly affected (Fig. 3E). The ovarian GSI showed a different pattern, with the 60IRC groups exhibiting a lower mean ovarian GSI than the other groups. This group, however, was only significantly lower than the 40NIRC and 60NIRC group averages. All the other diet treatment groups were not significantly different from each other (Fig. 3F).

3.4. Serum protein levels

Serum protein levels were examined in the CON, 40IRC/NIRC, and 60IRC/NIRC groups (the 20IRC/NIRC group was not analyzed due to insufficient resources). The protein treatment group averages of the levels ranged from 18.7 ± 2.2 to 31.7 ± 2.2 mg/ml protein, and were significantly decreased in the 40IRC and 60IRC diet groups compared to the CON diet (Table 2).

3.5. Vitellogenin levels

Because no standards were available for the vitellogenin analysis (serum from laying hens was used as a control), vitellogenin values could not be presented as amount vitellogenin per ml serum. Therefore, an average of the ratios is presented of the absorbency of the serum samples divided by each sample's protein level. Vitellogenin absorbency to protein level ratios ranged from 0 to 0.01, although the high value was an outlier in the 60IRC group, and removed from the analysis. There were no significant differences among the treatment groups, but the mean for the 60IRC group was higher than for the CON group (Fig. 4)

4. Discussion

4.1. Red clover estrogenic activity

It has long been known that red clover and other subterranean clovers contain estrogenic chemicals [35,40]. Early reports of infertility in livestock were attributed to estrogens in the pasture feed [41]. Red clover contains the phytoestrogens formononetin, biochanin A, and to a lesser extent, daidzein, and genistein [42–45]. Red clover was found to cause vaginal abnormalities and altered mating behavior in ewes [8], leading to permanent flock infertility [7], when ewes were fed red clover during periods in the first 4–5 years of their lives. Immature rats exhibited up to a 200% increase in uterine growth when fed 2 g red clover per day for 5 days [11].

Because of the interest in red clover as a potential supplement for the use in hormone replacement therapy for post-menopausal women [46], the estrogenic activity of red clover has been well studied. Although many studies have found that red clover extracts (of the whole plant or parts), are estrogenic using *in vitro* and *in vivo* techniques [42,46–49], few studies have quantified the *potency* equivalence to estradiol, and those that have are difficult to relate, due to differing extraction techniques, parts of the plant used, growing conditions, etc. For example, in a uterine growth bioassay, the estrogenic potency of red clover fed to mice was found to be less than 4 pmol E2 equivalence per gram plant [50], while an estimation of its potency in an *in vitro* MCF-7 proliferative assay was 2 nmol E2 equivalence per gram red clover blossom, and slightly higher for the red clover sprout [48]. Additionally, the type of bioassay used to determine potency must be taken into account. The BG-1 luciferase reporter gene assay used in our study acts primarily through the ER β estrogen receptor sub-type; the luciferase gene is present downstream of the ERE, so any ligand binding to the ER β in this assay will induce the production of luciferase in the cell. Thus, any actions of a compound downstream of the ERE or due to ER α cannot be quantified [39]. Many studies have examined the binding affinity of red clover extract to the estrogen receptor (ER) sub-types (alpha and beta), and the results vary widely. While one study found that red clover blossom and sprout extracts bind only to ER β [48], other studies have found that red clover extracts have equal and significant binding affinities to both ER α and ER β , as determined by competitive binding assays with estradiol [46,49]. Although whole diet rather than plant alone was analyzed in our study, the majority of the diet (60%) consisted of red clover (whole plant). The potencies for these diets fell within the wide range found in literature (between 7.0 and 49.4 pmol E2 equivalents per gram diet). While our potency estimates for red clover diet reflect the potency of the plant, additional studies including standardization of extraction techniques, bioassays and growing conditions are required to provide more accurate potency estimates for red clover.

Additionally, utilizing normalized BG-1 bioassay results, the control diets appeared to be more estrogenic than the red clover diets. This is, in fact, misleading. As shown in Fig. 1, the red clover diet extracts exhibited so-called “super-induction” of luciferase activity, compared to both the control diets and the estrogen standards. In essence, the maximum luciferase value of the red clover diets (which exceeded 12,000 RLUs in the bioassay) was as much as 3.5 times higher than the luciferase activity produced by a maximal inducing concentration of estrogen or the control diet (which did not exceed 4000 RLUs). Because of this, the red clover curves could not be directly compared to the estrogen standard curve. To directly compare the super-induced curves with the estrogen standard curve, the maximum RLU values of the standard curves and the diet curves were normalized to 100% of the maximal value, and EC₅₀ values of each curve were compared. This calculated value, however, only takes into account the action of the extract to stimulate the ER-dependent gene expression from the estrogen response element (ERE). The pattern of super-induction is consistent with enhanced transcriptional activity mechanisms downstream of the estrogen

response element, independent of or in addition to the ER activity, thus resulting in production of luciferase activity greater than the maximal induction produced by estradiol. Thus, when a chemical exhibits super-induction in these screening bioassays, a direct comparison of its potency to a standard curve is problematic. Plant chemicals in these complex mixtures could act in a number of ways to enhance estrogenic responses, independently and downstream of the ER and ERE. For example, they could act independently of the ER and ER ligands by enhancing transcriptional activity at the gene promoter and/or at the level of mRNA or protein. ER-dependent gene expression can be modulated by cell surface receptors [51], which could potentially be bound by phytoestrogens. Additionally, phytoestrogens can interfere with steroidogenic enzyme pathways [10,52], and have effects on protein phosphorylation and nuclear co-regulators [53].

The super-induction of the red clover diet extracts suggests that the diets can produce a greater and more effective ER-dependent response than the control diet (which did not super-induce). Additionally, direct comparisons of the red clover diets to the estradiol induction standard curve indicates they are more potent than the control diet. Due to the complicated actions of phytoestrogens in organisms, it is possible that the phytoestrogens in red clover act to induce estrogenic responses by enhancing recruitment or functionality of coactivating proteins, altering or inducing enzymatic responses, or otherwise stimulating estrogenic activity apart from and in addition to ERE binding. Unfortunately, in the scope of our study, it was impossible to assess the molecular mechanisms of action of the red clover extract responsible for the enhanced induction response. The indication of an alternate mechanism(s) of action may contribute to the difficulty of assessing the actual potency of red clover, and work elucidating the bioactive mechanism of red clover phytoestrogens needs to be done in order to gain an accurate estimate of its estrogenic potency.

One aim of our study was to determine whether drought-stress increased phytoestrogens or estrogenic activity in red clover. Stressors do, generally, increase plant secondary compound production (e.g., heat stress induces the production of phytoecdysteroids [54] and drought-stress induces other secondary metabolites [55]). Further, Leopold et al. (1976) found greater levels of phytoestrogens in the vegetation eaten by California quail in a year with less rainfall than in two other years of heavy rainfall [31]. Many studies have examined factors that affect the estrogenic activity and/or isoflavone content of red clover, as well as potency differences among plant parts. Tsao et al. (2006) found that the highest concentrations of isoflavones were present in the leaf, followed by the stem, petiole, and finally the flower. Further, they found that the concentration of isoflavones increased from the early bud stage, to the late flowering stage in the leaves, while the isoflavone concentration in the stems and petioles decreased as the plants matured [44]. Another study also found decreases in formononetin content in the later growing stage, and that decreases in temperature increased formononetin content in leaves [45]. Booth et al. (2007) found that flower heads had less isoflavones and lower estrogenic activity than other above ground parts. They also found that specific isoflavones had different peak concentrations at different times of year, and some evidence that increased rainfall corresponds to increased estrogenic activity [46], which is possibly consistent with our study. UV-B radiation has been found to induce plant secondary metabolite production [56], and Swinny and Ryan (2005) found that UV-B induces formononetin and biochanin A in red clover [43]. This fact is particularly interesting, as it has been postulated that phytoestrogens may have evolved as plant defenses against photo-damage [57].

In our study, the irrigated red clover (grown between October and April) was more potent than the non-irrigated red clover (grown between April and June), according to the BG-1 luciferase assay. This would appear to contradict our hypothesis that drought-stress induces

the production of phytoestrogens (but is consistent with the findings of Booth et al. [46]). The age of the plants may have affected the phytoestrogen content, as the later harvest corresponded to older red clover plants. The estrogenic activity of older red clover plants compared to younger plants was not analyzed in our study, however. Additionally, although not quantified, it was clear from visual inspection that the irrigated red clover had more leaf matter than the non-irrigated red clover, which was judged to be of “poor quality” by an expert hay cultivator and it also had a higher proportion of stems and buds. Due to the fact that the leaves of red clover contain a higher content of estrogenic isoflavones than stems and flowers [44,46], it is possible that the lower estrogenic activity of the non-irrigated diet in our study was due to the lower amount of leaf matter in the vegetation.

4.2. Chick weights

Chicks eating both the irrigated and non-irrigated diets showed stunted growth in our study. Because food consumption was not monitored, it is not known whether the decrease in growth in our study was due to decreased food consumption of the higher concentration-red clover diets. Food dishes were refilled more often in the control and lower concentration red clover diets, but increased food consumption may have been due to the increased weight of the control and low-dose animals. Phytoestrogens administered during development have been shown to decrease growth in rodents, both post-natally [58] and prenatally [59]. Rats fed on soy-containing vs. soy-free commercial diets throughout life also showed reduced bodyweights as adults [60], but this trend was reversed in another study in rats [9]. Additionally, ewes had greater bodyweights when consuming red clover pasture than when consuming a ryegrass/white clover pasture mix [7]. In birds, diets containing the phytoestrogens coumestrol or biochanin A had no effect on body mass growth in scaled quail [32], but broilers consuming soy isoflavones in feed had reduced feed intake and bodyweight [61]. Due to the severe decrease in growth rate seen in our study, it is probable that this decrease in body mass was, in part, due to a decrease in food consumption in the higher content red clover diet treatments, in addition to possible physiologic effects of the red clover on growth.

4.3. Gonad weights

Both the absolute weight and the gonadosomatic index (GSI) of testes were decreased in male Japanese quail consuming diets high in both irrigated and non-irrigated red clover. Decreased testes growth in response to phytoestrogens has been documented in rodents, with rats fed on soy-containing commercial diets during development having decreased testes weights and decreased luminal areas as adults, and genistein inducing these effects in soy-free diets [60]. Additionally, rats fed a genistein supplemented diet during development had decreased sperm counts as adults [62]. Adverse effects of phytoestrogens on testes have also been found in fish [63] and frogs [64]. Few studies have examined the effects of phytoestrogens on avian testes, but Opalka et al. (2006) found that various phytoestrogens inhibited testosterone production in cultured Bilgoraj gander Leydig cells [65].

Although the absolute oviduct weights were decreased with increasing concentrations of red clover in the diet, GSI of the oviducts was increased in the 60IRC group. This elevation corresponded with the irrigated red clover diet. Uterotropic assays are commonly used in rodents to assess estrogenic activity [66,67]. Similarly, estrogen exposure induces oviduct growth in birds [68], and phytoestrogens have been shown to induce oviduct growth in rodents [67] and birds [13,14]. Previous studies in our laboratory have tested the oviduct-growth response of zebra finch chicks by orally exposing chicks to 0.1–1000 nmol estradiol benzoate per g bodyweight per day for a week. Additionally, the exposure of E2 to the chicks corresponded with decreased fertility as adults. The chicks exposed to the low dose (0.1 nmol EB per g BW) exhibited a two-fold increase in oviduct weight [13].

Several studies have shown contradictory evidence, finding that phytoestrogens do not induce oviduct growth in quail, either in a soy isoflavone-enhanced diet [69] or with exposure to coumestrol or biochanin A added to diets [32]. Although these studies indicate that isolated phytoestrogens may not affect reproductive endpoints in quail, they were not whole diet studies, in which combinations of phytoestrogens may act to enhance the estrogenic effect on vertebrates.

Lastly, ovary absolute weights and GSI were significantly decreased in the most potent diet group (60IRC) in our study. While exposure to exogenous estrogen during development has been shown to disrupt ovarian histology in birds [70], embryonic estradiol benzoate exposure does not appear to decrease ovarian mass in Japanese quail in previous studies [71]. However, the potent synthetic estrogen, diethylstilbestrol (DES), decreased ovarian weight in adult scaled quail [32]. Additionally, several studies in fish have shown that exogenous estrogens inhibit GSI mass during gonadal recrudescence [72,73]. It is unclear if the reduced ovary mass seen in our study is due to the estrogenic properties of the diets, and this area merits further investigation.

4.4. Vitellogenin

Vitellogenin levels were not significantly different in any of the diet treatment groups in our study, and were widely variable in each group, although the highest outliers recorded were seen in the 40IRC and 60IRC diet groups. Vitellogenin is a yolk protein, produced by the liver of oviparous vertebrates in response to estrogen, and transported to the egg yolk via the bloodstream [74]. In males and pre-pubertal animals, serum vitellogenin levels can indicate exogenous estrogen exposure [75,76]. Although the vitellogenin bioassay has been suggested as a sensitive endpoint for environmental estrogen exposure in Japanese quail, with significant elevations of vitellogenin at 10 ppm E2 exposure levels [70], our study indicates that other endpoints (such as oviduct growth) are more sensitive when studying exposures to low potency exogenous estrogens. Indeed, other studies have indicated that vitellogenin levels are too variable to use as a sensitive endpoint for estrogen exposure in Japanese quail [77].

4.5. Ecological implications

Leopold et al. (1976) postulated that the signal which initiated reproduction in California quail in arid habitats may be levels of phytoestrogens in plants, which are variable depending on the growing conditions [31] and this hypothesis has received some support. Labov (1977) suggested that red kangaroo reproduction is initiated by changes in phytoestrogen levels in grasses [15], and others have more recently postulated that hormonal chemical plant signaling may regulate vertebrate reproduction [16,17]. Cain et al. (1987) studied the effects of specific phytoestrogens (coumestrol and biochanin A) on adult scaled quail and found that only very high levels added to the diet affected reproductive parameters (50 mg/day coumestrol induced slight oviduct growth, and 10 mg/day biochanin A decreased fertility). They stated that, although an effect was found, quail would not be exposed to this level of phytoestrogens in the wild, and thus the theory of Leopold and coworkers was not supported [32].

Although the studies of Cain et al. (1987) may indicate that there are no ecological effects of phytoestrogens on vertebrates in the wild, their study does not rule out the role of phytoestrogens as an environmental regulator of reproduction. These authors only exposed birds to a few specific phytoestrogens, rather than the whole plant diet, which contains many types and combinations of estrogenic isoflavonoids and other chemicals [43]. It is known that combinations of environmental estrogens can have additive effects on endocrine disruption [78]. Additionally, Cain and coworkers only studied the activational effects of

phytoestrogens on reproductive endpoints. While Leopold et al. [31] did indeed suggest that phytoestrogens have an activational role in reproductive signaling, the organizational effects of exogenous estrogens are often more sensitive end-points [13], and it is possible that phytoestrogens in the wild exert more of an effect on vertebrates during development. Phytoestrogens have been shown to accumulate in the yolk of Japanese quail via maternal transfer [79], and phytoestrogen exposure during development may alter adult reproduction [13]. These facts, combined with the estrogenic effects documented in our study, suggest that we cannot rule out the hypothesis that plant phytoestrogens exert a reproductive effect on wildlife, especially on developing vertebrates. Additionally, while we did not find that drought stress increased the estrogenic activity of the diets, this may have been due to the lack of foliage on the drought-stressed plants, or the real possibility that drought stress may decrease the estrogenic potency of red clover.

4.6. Summary

There is evidence to suggest that plant-produced phytoestrogens in the diet of wild birds exert a reproductive effect, allowing birds to adapt to changing environmental conditions. We found that: (1) formulated red clover diets (with red clover grown in irrigated and non-irrigated conditions) were estrogenic, with irrigated red clover diets being more potent than non-irrigated diets; (2) the diets caused a decrease in bodyweight in Japanese quail chicks; and (3) the diets caused a decrease in absolute gonad weights, a decrease in relative testes and ovary weights, yet an increase in oviduct weight in chicks. The red clover diets had no effect on serum vitellogenin levels, indicating that gonad weights are a more sensitive endpoint of environmental estrogen exposure than vitellogenin induction. Our study suggests that environmental conditions can, in fact, change the estrogenic potency of red clover, and that high levels of red clover can affect growth and sexual development in birds.

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Fig. 1.

Estrogenic potency curves of estradiol standards and diet extracts. Relative luciferase unit (RLU) induction curves from the BG-1 luciferase bioassay of (A) 17 β -estradiol standards and (B) control diet (CON), 60% irrigated red clover diet (60IRC), and 60% non-irrigated red clover diet (60NIRC), showing super-induction of the red clover diets. The dashed lines indicate EC₅₀ values of the estradiol standards and diet extracts.

Fig. 2.

Growth of Japanese quail chicks fed on irrigated and non-irrigated red clover diets. Mean ($\pm$ S.E.) bodyweights of Japanese quail chicks fed on 20%, 40% or 60% irrigated or non-irrigated red clover diets (20IRC/NIRC, 40IRC/NIRC, 60IRC/NIRC) or a control diet (CON), from post-hatch Day 1 to post-hatch Day 15. $N = 3$ pens per diet group, with a range of 7–12 individuals per pen. Letters indicate significant differences: (a) significantly different from control ($p < 0.05$), (b) significantly different from corresponding non-irrigated diet ($p < 0.05$), as determined by ANOVA, PROC MIXED.

Fig. 3.

Absolute gonad weights and gonad GSIs of Japanese quail chicks fed on irrigated and non-irrigated red clover diets. Mean ($\pm$ S.E.) absolute gonad weights and GSI of Japanese quail chicks fed on 20%, 40% or 60% irrigated or non-irrigated red clover diets (20IRC/NIRC, 40IRC/NIRC, 60IRC/NIRC) or a control diet (CON), from post-hatch Day 1 to post-hatch Day 15, measured on post-hatch Day 15; (A) absolute testes weights, (B) testes GSI, (C) absolute oviduct weights, (D) oviduct GSI, (E) absolute ovary weights, and (F) ovarian GSI. $N = 3$ pens per diet group, with a range of 7–12 individuals per pen. Letters indicate significant differences: (a) significantly different from control ($p < 0.05$), (b) significantly

different from corresponding non-irrigated diet ($p < 0.05$), and (c) significantly different from diets with an asterisk (*) above them, as determined by ANOVA, PROC MIXED.

Fig. 4.

Relative serum vitellogenin levels of Japanese quail fed on irrigated and non-irrigated red clover diets. Mean ($\pm$ S.E.) of the ratio of relative serum vitellogenin levels to serum protein levels of Japanese quail chicks fed on 40% or 60% irrigated or non-irrigated red clover diets (40IRC/NIRC, 60IRC/NIRC) or a control diet (CON), from post-hatch Day 1 to post-hatch Day 15, measured on post-hatch Day 15. Relative serum vitellogenin of each individual was measured in absorbency (nm) and divided by each individual's serum protein level (mg/ml) to obtain the final ratio. *N* represents number of individuals. *N* = 10 in CON, 40IRC, 40NIRC, and 60NIRC. *N* = 8 in 60IRC. No significant differences were detected, as determined by ANOVA, PROC GLM.

Table 1

Potency estimates of control and red clover diet extracts. Potency estimates of control diet extract, 60% irrigated red clover diet extracts (60IRC), and 60% non-irrigated red clover diet extract (60NIRC), determined using a BG-1 luciferase reporter gene assay, at EC₂₅ and EC₅₀ of the estrogen standard and samples. Potencies are reported as pmol 17 β -estradiol (E2) equivalents per gram diet.

<i>Diet</i>	Potency Estimate (pmol E2 equivalence per gram diet)	
	EC ₂₅	EC ₅₀
Control	62.5	40.0
60IRC	49.4	20.2
60NIRC	10.0	7.0

Table 2

Serum protein values of Japanese quail chicks fed on irrigated and non-irrigated red clover diets. Mean ($\pm$ S.E.) serum protein levels (mg/ml) of Japanese quail chicks fed on 20%, 40% or 60% irrigated or non-irrigated red clover diets (20IRC/NIRC, 40IRC/NIRC, 60IRC/NIRC) or a control diet (CON), from post-hatch Day 1 to post-hatch Day 15, with protein measured on post-hatch Day 15. *N* represents number of individuals.

<i>Diet</i>	<i>N</i>	Serum protein (mg/ml)
Control	10	31.7 $\pm$ 2.2
40IRC	10	22.0 $\pm$ 2.2 *
60IRC	8	18.7 $\pm$ 2.2 *
40NIRC	10	28.3 $\pm$ 2.4
60NIRC	10	26.8 $\pm$ 2.2

The asterisks (*) represent values significantly different from CON at $p < 0.05$ as determined by ANOVA, PROC GLM.

* $p < 0.05$, ANOVA.