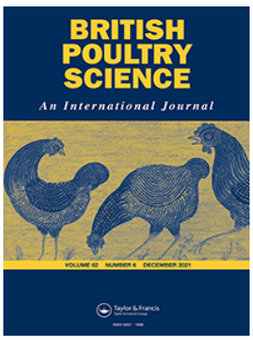

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Red clover-rich grassland increases equol concentration in eggs from free-range laying hens

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ABSTRACT

1. The aim of this study was to evaluate the production of equol (4′,7-isoflavandiol; a bacterial polyphenol metabolite which is an isoflavandiol oestrogen metabolised from daidzein from plants) enriched eggs from free-range hens fed different pasture species. Four species were tested: red clover, white clover, ryegrass and chicory.
2. The study was conducted from June to September 2017 on eight free range, outdoor areas, each containing fifteen laying hens and sown with a single pasture species
3. Precursors of equol (daidzein, formononetin) were analysed every fortnight from the fresh pasture cover in each area, as well as equol and daidzein levels in eggs.
4. Daidzein and formononetin concentrations in the fresh pasture samples differed significantly according to species ($P < 0.001$), whereby red clover had the highest concentrations of daidzein and formononetin (85 and 996 µg/g DM, respectively).
5. Equol concentration in eggs differed according to pasture species ($P < 0.001$). Equol concentrations reached about 1,200 ng/g DM in eggs from hens with access to red clover. These eggs can represent a valuable source of equol in the human diet.

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Equol; egg quality; free-range; pasture; laying hen

Introduction

For some years, equol, (4′,7-isoflavandiol) an isoflavandiol oestrogen metabolised from plant material by gut bacteria, has attracted the attention of scientists. This metabolite results from microbial processing of specific dietary isoflavones (daidzein, formononetin) in the digestive system of humans and animals (Setchell et al. 2002). Equol has been described having positive effects on human health, in terms of prevention or treatment of certain diseases, such as hormone-dependent cancers, cardiovascular diseases and osteoporosis (Setchell and Clerici 2010; Jackson et al. 2011; Legette et al. 2014). A study of healthy Asian people who were equol producers (i.e. able to transform daidzein and formononetin into equol through their gut microbiome) indicated that a concentration of 5 to 10 ng of equol per ml of blood plasma may lead to benefits for human health (Jackson et al. 2011). However, only a small proportion of the Western population is able to produce equol due to a lack of appropriate bacterial activity in the digestive system (Yuan et al. 2007; Mayo et al. 2019).

Eggs could be a good way to provide equol in the human diet, as they have a high nutritional value and low-cost production (McNamara 2015; EC 2018). The albumen and yolk contains 76.1 g of water per 100 g and supplies about 12.5 g and 9.5 g of proteins and lipids per 100 g (Réhault-Godbert et al. 2019). The egg amino acid profile matches well with human nutrient requirements (Herron and Fernandez 2004; Nau et al. 2010; Miranda et al. 2015). When hens are given a conventional feed based on corn, wheat and soya bean, 60% of the total fatty acids in eggs are polyunsaturated (Nau et al. 2010). Moreover, eggs are a good vehicle for additional nutrient supply within the food chain (Rooke et al. 2010; Mugnai et al. 2014). For example, Cherian and Quezada (2016) compared the composition of eggs obtained from hens fed a diet containing 10% flaxseed to those from a corn-soya bean control diet. The total lipid content of egg yolks did not show any significant difference, while the egg yolk fatty acid profile was significantly different, with 60 mg of total omega-3 fatty acids per egg for the control and about 150 mg per egg for the flaxseed group. In addition, studies on the impact of organic source of selenium in laying hens' diet on egg content showed that 30 µg per egg could be attained, representing 50% of the daily human requirement (Fisinin et al. 2009). The ease with which the nutritional properties of eggs can be changed is a useful feature, given that consumers are looking for healthier food, but may not wish to change their eating habits (Martínez et al. 2012). According to Saitoh et al. (2001), equol can be expressed in the yolk of eggs by adding purified isoflavones, extracted from soya bean hypocotyl, to laying hen diets.

A further aspect to consider is consumer expectations in terms of animal welfare and production methods (EC 2018). Livestock production systems have changed whereby, in Europe, between 2007 and 2018, the share of non-battery egg production methods increased from 25 to 50%, and outdoor, free range production now represents 16% of egg production (European Commission, 2019). Free-range access to pasture can lead to enrichment of egg with various nutrients, as it has been established that hens incorporate a significant proportion of pasture plant material in their diet (Mugnai et al. 2014).

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Considering consumers' expectations for healthier food and production methods including animal welfare, the following study was designed to evaluate if equol-enriched eggs could be produced from free-range hens. The second objective was to assess the effect of pasture species (containing different levels of isoflavones) sown in outdoor free range areas on the equol content of their eggs, when provided for six months. According to current literature, few studies on the production of equol-enriched eggs have been published.

Materials and methods

Experimental design

The study was conducted at the Walloon Agricultural Research Center (Gembloux, Belgium) from June to September 2017. Eight free range areas were included in the experimental design and were separated with chicken wire, avoiding the movement of laying hens from a plot to another. Each plot contained a henhouse (3.5 x 3 m) and a pasture area (17.5 x 6 m). Each henhouse included a feeder, a drinking trough and five nesting boxes. The henhouse floor was covered with a wood-chip litter. There was natural light from a window, and neither artificial lighting nor heating were installed. An automatic trapdoor was placed on each henhouse, allowing laying hens to have access to outdoor areas from dawn until dusk each day.

In late winter 2017, existing pasture was sprayed off (Roundup©, 6 l/ha) and the soil was prepared for sowing followed by a second herbicide treatment. Four pasture species were tested; red clover (RC, *Trifolium pratense* L.), white clover (WC, *Trifolium repens* L.), chicory (CH, *Cichorium intybus* L.) and ryegrass (RG, *Lolium perenne* L.). Each species was sown on two randomly selected outdoor areas. During the trial, pasture cover was maintained in a vegetative state thanks to regular mowing, which was individually adjusted to prevent flowering, depending on the climatic conditions.

The laying hens were 17 weeks old at the start of the experiment. The strain NovoGen Brown Light was chosen for its suitability for free range production. A total of 120 laying hens were sourced from the same farm and randomly distributed in groups of 15 to each pasture treatment. As soon as the hens arrived, they were given a conventional feed balanced for outdoor production, offered *ad libitum* which was used throughout the study. The ingredients and chemical composition of the balanced feed are shown in Table 1.

Table 1. The balanced feed for outdoor production offered *ad libitum* to laying hens during all the study: ingredients and chemical composition (calculated values).

	Feed
Ingredient, g/kg DM	
Wheat	340
Maize	300
Soybean meal	200
Crushed limestone	60
Soybean oil	40
Bran	25
Chalk	23
Vitamins and minerals	12
Chemical composition	
DM, g/kg	890
Crude protein, g/kg DM	160
Fat, g/kg DM	53
Ash, g/kg DM	120
Starch, g/kg DM	430
Calcium, g/kg DM	35
Phosphorus, g/kg DM	4

Animal performance

Eggs were collected every day from the nest boxes and from the outdoor plots and were counted and weighed. The laying rate (number of eggs per hen per day) was calculated per fortnight for each pasture treatment. The average weight of the eggs was calculated by dividing the total weight of eggs by the number of eggs collected on each plot during the fortnight.

Sample collection

Each fortnight, pasture cover and eggs were sampled. For each treatment, pasture cover was cut at ground level in seven random areas throughout the plot to provide a composite sample weighing about 200 g of fresh matter. A sample of the compound feed was collected at the beginning and the end of the study. All samples (pasture cover, balanced feed) were stored at −20°C, freeze-dried at 850 mbar with a lyophiliser (Martin Christ Delta 1–24 LSC plus, Analis, Suarlée, Belgium) and ground in a Cyclotec mill (1 mm screen, FOSS, Hillerød, Denmark). They were vacuum-packed with a vacuum packaging machine (model UNICA GAS, Lavezzini, Fiorenzuola, Italy) and stored at −20°C before analyses. On the day of the collection, eggs from the same plot were broken individually and separated. Egg yolks were mixed and stored at −20°C while egg whites were discarded.

The study ran for 20 weeks in fortnightly blocks, and ten samples of fresh pasture cover and ten samples of eggs were collected from each plot. In total, 81 vegetal samples were analysed, comprising 80 samples of fresh pasture cover and one pooled sample of the balanced feed, and 80 egg yolk samples were analysed.

Sample preparation

Diet samples were analysed in duplicate using a method validated by Daems et al. (2016) for the quantification of equol precursors (daidzein, formononetin), with recovery rates in the acceptable range of 70–120%. First, methanolic ultrasound-assisted extraction was used to remove the target analytes from the plant matrix, whereby 25 ml of H_2O: methanol (45:55, v/v) was added to 0.5 g of the sample, which was then placed in an ultrasonic bath over 10 min at 80°C. After centrifugation at 3,200 x *g* for 5 min, the supernatant was evaporated to dryness and then solubilised with 1 ml of sodium acetate buffer (pH 6). Secondly, enzymatic hydrolysis was performed to obtain aglycones from conjugated forms, whereby 2 ml of an enzymatic solution was added, including β-glucosidase (from almonds, ≥ 12 units/ml, 9001–22-3), β-glucuronidase (type H-2 from *Helix pomatia*, ≥ 5,1000 units/ml, 9001–45-0) and cellulase (from *Aspergillus niger*, ≥6 units/ml, 9012–54-8) dissolved in 0.2 M of sodium acetate buffer (pH 6). All enzymes were purchased from Sigma-Aldrich (Diegem, Belgium) and hydrolysis was performed over 18 h at room temperature. Post-hydrolysis steps were taken to recover target compounds, and several dilutions ensured that the sample concentration lay in the range of quantification. After a second

centrifugation (3,200 x *g* for 5 min), the supernatant was collected. Several dilutions (dilution factor of 10, 50 and 100) with H_2O:methanol (40:60, v/v) were evaporated to dryness and recovered with 1 ml of injection standard solution. The injection standard solution included 50 and 20 ng/ml of daidzein-d4 and flavone, respectively, diluted in H_2O:methanol (40:60, v/v). Daidzein-d4 (1219803–57-2; C-D-N ISOTOPES, Pointe-Claire, Canada) and was used to quantify daidzein. The flavone 'IS' (525–82-6; Sigma-Aldrich, Diegem, Belgium) was used to quantify formononetin and the recovered solution was filtered (0.2 µm) and injected into the ultra-performance liquid chromatography coupled with a tandem mass spectrometry (UPLC®-MS/MS) system.

Egg samples were analysed in triplicate to quantify equol and daidzein using methods adapted from Saitoh et al. (2001) and Abiru et al. (2012). However, the method and the material used did not allow the quantification of the formononetin in egg yolk. The recovery rate of equol and daidzein were estimated by the standard addition method and were within acceptable range, and so an extraction standard was not used. About 3 g of thawed yolk was mixed with 12.5 ml of 0.2 M sodium acetate buffer (pH 6) and 100 µl of β-glucuronidase (type H-2 from Helix pomatia, ≥85,000 units/ml; 9001–45-0). The enzymatic hydrolysis was conducted over 18 h at 37°C with gentle stirring. The first extraction of the target compounds was performed with 15 ml of ethanol. After centrifugation (3,000 x *g* for 5 min), the supernatant was recovered and stored at −20°C overnight. The next day, once the solution had been brought to room temperature, 10 ml of clear supernatant was collected and added to 20 ml of H_2O:hexane (50:50, v/v) in order to remove fat, which is a major cause of variation in tandem mass spectrometry (MS/MS) analysis. The solution was centrifuged (3,000 x *g* for 5 min) and the supernatant discarded. A double extraction (20 and 15 ml, respectively) with ether was performed to recover the aglycone forms. Supernatants from each treatment were pooled and mixed in the same tube, evaporated to dryness and recovered with 1 ml of injection standard solution (20 ng/ml of flavone). The solution was filtered (0.2 µm) and injected into the UPLC®-MS/MS system.

For both types of samples, external calibration was done with concentrations from 5 to 200 ng/ml ($R^2 \geq 0.99$) for the target compounds in solution diluted in H_2O:methanol (40:60, v/v). The limits of quantification (LOQ) were 1.6 µg/g of DM and 2 ng/g of DM for the pasture samples and the egg yolks, respectively. The formononetin and equol standards were acquired from Sigma-Aldrich (Diegem, Belgium), and the daidzein from Cayman Europe (Tallin, Estonia).

LC-MS/MS analysis

The LC analyses were conducted on an ACQUITY UPLC from Waters (Zellik, Belgium). The column used was a Waters ACQUITY UPLC HSS T3 (2.1 x 100 mm, 1.8-µm particle size) with a preconnected in-line filter (0.20 µm). The column was kept at 45°C and the vials at 4°C, and an aliquot of 10 µl was injected. The mobile phases consisted of ultrapure water with 0.1% formic acid (eluent A) and methanol with the same percentage of acid (eluent B).

The eluting compounds were detected using a Waters TQ Detector (Micromass Waters, Zellik, Belgium) with an electrospray ionisation interface. The mobile phase flow rate was 200 µl/min. The optimal source and desolvation temperatures were 140 and 350°C, respectively. Cone gas and desolvation gas flow (both N_2) were set at 50 and 700 l/h, respectively. Argon was used for the collision-induced fragmentation. All the data were collected and processed using the MassLynx software with a Quanlynx program (Micromass Waters, Zellik, Belgium).

Statistical analysis

Statistical analyses for both animal performance and analytical results were performed with Rstudio software (version 3.6.1, R Core Team, Vienna, Austria). Analyses of variance, including the two fixed factors (pasture species and time) and one random factor (plot) were implemented with function lm. Each plot was designated as an experimental unit, and the time period for measures was the fortnight. Comparisons of means were executed with the Tukey test in the glht function (multcomp package). P-values equal to or less than 0.05 was deemed significant.

The number of replicates was equal to two in view of the exploratory character of the research, the number of samples collected and the equipment required. Two replicates were considered as sufficient in the present study design, considering that (i) fifteen laying hens were used in each replicate (decreasing dividual variability) and (ii) repeated measurements were performed for six months to confirm the effect of pasture species over six months.

Results

Animal performance

Laying performance data is presented in Table 2. Across all treatments, the mean laying rate was 0.868 and the mean egg weight about 60 g. Statistical analyses of laying rate and egg weight did not show either an interaction between pasture species and time, or a significant effect of pasture species. A time effect was seen for both parameters, whereby laying rate was significantly lower in the first fortnight (0.65) after which it reached a plateau for the rest of the study (from 0.87 to 0.93). The egg weight increased at the beginning of the study, being significantly different each fortnight from May 31 (with an average weight of 48.3 g) to July 12 (with an average weight of 60.7 g). After this date, only minor differences were observed from July 6 to October 4, although the last fortnight showed the highest average egg weight (64.0 g). During the study, one hen died in WC treatment. This death was taken into account in the calculation of laying rate.

Isoflavone and equol concentrations

The concentrations of targeted compounds are shown in Table 3. For the fresh pasture cover, there was no difference in daidzein and formononetin concentrations over time. However, RC had the highest concentrations, which were two to three times higher than in WC. Concentrations were low in RG and only trace levels (<LOQ) were found in CH. In addition, to characterise all the distributed feed to laying hens, the contents of equol precursors were also analysed in the balanced feed and reached 150 µg/g DM of daidzein while formononetin was below the LOQ.

Table 2. Impact of grassland species and time on laying rate (egg/hen/day) and egg weight (g).

	Grassland species					P-value		
Item	RC[1]	WC[2]	CH[3]	RG[4]	SEM[5]	G[6]	T[7]	G*T
Laying rate (egg/hen/day)	0.816	0.871	0.880	0.906	0.039	0.273	< 0.001	0.094
Egg weight (g)	60.3	60.1	58.9	59.8	0.54	0.171	< 0.001	0.770

[1]RC, red clover; [2]WC, white clover; [3]CH, chicory; [4]RG, ryegrass; [5]SEM, standard error of the mean; [6]G, grassland species, [7]T, time.

studies. Daems et al. (2016) reported 2,051 µg/g DM for formononetin and 127 µg/g DM for daidzein and Adler et al. (2014) found from 1,539 to 3,855 µg/g DM for formononetin and from 13.3 to 28.5 µg/g DM for daizein in fresh red clover. Andersen et al. (2009) found concentrations reaching 11,420 µg/g DM formononetin in fresh RC. These variations can be explained by genetic, plant-stage, seasonal and environmental factors (Lemežienė et al. 2015). For instance, formononetin concentration is usually higher in leaves than other parts of the plants and decreases after the onset of flowering

Table 3. Impact of grassland species and time on concentrations of target compounds in fresh grassland cover and in egg yolk.

	Grassland species					P-value		
Item	RC[1]	WC[2]	CH[3]	RG[4]	SEM[5]	G[6]	T[7]	G*T
Fresh grassland cover, µg/g DM								
Daidzein	85[a]	41[b]	< LOQ[8,c]	5[c]	10	< 0.001	0.177	0.988
Formononetin	996[a]	282[b]	< LOQ[d]	20[c]	78	< 0.001	0.774	0.948
Egg yolk, ng/g DM								
Equol	1,154[a]	282[b]	109[d]	229[c]	41	< 0.001	0.081	0.365
Daidzein	661[a]	228[b]	59[d]	175[c]	41	< 0.001	0.004	0.883

[1]RC, red clover; [2]WC, white clover; [3]CH, chicory; [4]RG, ryegrass; [5]SEM, standard error of the mean; [6]G, grassland species, [7]T, time; [8]LOQ, limit of quantification. [abcd]In the same row, means with different superscript letters indicate a significant difference between grassland species.

Daidzein and equol in egg yolks was affected by pasture species. Access to RC led to the highest concentrations, which were four and three times higher than in WC, for equol and daidzein, respectively. Birds with access to RG and CH had only low concentrations, and a time effect was significant for daidzein but not for equol. Daidzein concentration was the lowest on May 31, at the beginning of the study (162 µg/g DM). An intermediate level was observed on June 14 (241 µg/g DM), and the highest concentrations were achieved from June 28 to July 26 (from 334 to 399 µg/g DM). Intermediate levels were then observed at the end of the study (from 250 to 281 µg/g DM).

Discussion

The animal performances observed was close to the expected level for this strain, indicating a laying rate between 0.85 and 0.93 egg/hen/day from the age of 23 weeks (Novogen Online Technical Data, 2019). No statistical effect of pasture species was seen for animal performance, which may be attributed to the low number of replicates. A study with more replicates could confirm these results. However, regarding other studies, Horsted et al. (2006) had similar results using another strain with free-range access and fed a balanced diet, achieving a mean laying rate of 0.91 egg/hen/day and an egg weight of 59.6 g/egg, without any significant effect of the pasture species (red clover/ryegrass *vs.* forbs). Gjorgovska et al. (2016) fed laying hens added isoflavones (1,000 mg/kg) and observed an increase of 0.08 in the laying rate compared to the control group (0.80 *vs.* 0.72), but no further increase was seen with the addition of greater amounts. In contrast, the performance of other animal species have been decreased by ingestion of red clover due to its high phytoestrogen content, in particular in sheep due to fertility problems (Hashem and Soltan 2016).

Concerning equol precursors in fresh pasture, RC showed, as expected, the highest concentrations for formononetin and daidzein, although the levels were lower than seen in other (Sivesind and Seguin 2005; Booth et al. 2006). The concentrations of formononetin and daidzein were low for WC and RG and mostly under the LOQ for CH, as in other studies (Andersen et al. 2009; Adler et al. 2014).

In eggs, daidzein and equol concentrations were the lowest from hens with access to CH (59 and 109 ng/g DM, respectively). These results indicated that soya bean in feed as the only source of equol precursors was not sufficient to obtain a significant quantity of equol in eggs. Conversely, eggs laid by hens whose diet included RC showed the highest concentration of equol, averaging 1,154 ng/g DM. Assuming that one egg yolk weighed 9.3 g DM (17 g of fresh matter, with 55% DM), this result meant that one fresh egg from hens with access to RC contained about 11 µg of equol. Saitoh et al. (2004) gave hens a feed supplemented with purified isoflavones extracted from soya bean hypocotyl. Among the different isoflavones, the only equol precursor was daidzin, the glycoside form of daidzein. The experimental diet containing 380 µg/g daidzin led to an equol concentration estimated at 472 ng/g in fresh egg yolk, which corresponded to about 8 µg per egg. This comparison indicated that an egg-producing strategy combining a balanced feed and free access to fresh RC led to a similar equol egg content than a strategy based on supplements of purified isoflavones. Considering that a concentration of 5 to 10 ng/ml of equol in blood plasma has been associated with benefits for human health, the equol-enriched eggs produced in this study might be considered as a valuable source.

Data regarding the bioavailability of equol from eggs, as well as on the clearance rate of plasma equol, are needed to establish the benefit of equol-enriched eggs for the consumer. For that purpose, a nutritional trial on human volunteers consuming equol-enriched eggs on a regular basis should be designed. Moreover, the equol content in eggs may be further increased. Saitoh et al. (2004) tested a second experimental diet with 112 µg/g of daidzin in the diet, and obtained an equol content of 25 µg per egg. This suggested that the equol content of eggs can be raised by

increasing the dietary intake of its precursors. In the present study, the correlations (R^2) between equol concentration in eggs and formononetin or daidzein in fresh pasture cover were 0.80 and 0.64, respectively. These results indicated a high correlation between equol in eggs and formononetin from pasture cover. An easy way to increase the equol content in eggs could be to increase formononetin in fresh red clover by choosing varieties with a higher concentration. A complementary approach may be to provide the hens with minced RC, which may facilitate the bacterial access to the precursors of equol in the digestive tract of the animals.

To conclude, this preliminary study showed that equol-enriched eggs could be produced by free-range hens. The choice of the pasture specie sown in free range areas seemed to be important, with red clover showing the highest equol content in egg yolk. Further studies are needed to confirm these results, particularly considering the impact of pasture species on animal performance. Another point to explore is the processing of precursors into equol in the digestive tract of hens in order to optimise the factors stimulating this conversion. In addition, animal variability should be studied by performing trials on individual animals and analysing the digestive transformation of precursors into equol and the efficiency of transfer to eggs.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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