



Effects of insect herbivory on phytoestrogen concentrations in three *Trifolium* species

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Abstract

Phytoestrogens are plant-produced secondary compounds believed to play a protective role against environmental stress. When consumed by animals, phytoestrogens can influence animal health and reproductive function; hence, there is interest in identifying factors that trigger their production in agricultural crops. We conducted two experiments to determine if leaf tissue herbivory by corn earworm (*Helicoverpa zea* Boddie) induces phytoestrogen accumulation in three clover species commonly utilized as livestock forage. In experiment I, insects were fed on red clover (*Trifolium pratense* L.) and kura clover (*T. ambiguum* L.) for 7 days. Herbivory increased total phytoestrogen levels by 112% in red clover, while levels in kura clover were extremely low and not influenced by herbivory. In experiment II, herbivory was applied to two red clover cultivars and one white clover cultivar (*T. repens* L.) for 9 days followed by a 9-day recovery period or for 18 days without recovery. After 9 days of herbivory and a 9-day recovery period, total phytoestrogen levels in red clover increased by 84%. Compared to the control, there were no differences in total phytoestrogen concentration when herbivory was applied to red clover for 18 days; however, additional sampling revealed concentrations were over 112% higher in undamaged compared to insect damaged tissues. Herbivory did not influence total phytoestrogen concentrations in white clover. These findings highlight the role insect herbivory can play in modifying the phytoestrogen status of red clover, and therefore a potentially novel mechanism by which insect herbivory may indirectly influence agricultural livestock health and reproductive function.

Keywords Red clover · White clover · Kura clover · *Helicoverpa zea* · Isoflavones · Phytoestrogens

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Introduction

Some species of perennial legumes used as forage for livestock have the ability to produce phytoestrogens, plant-derived secondary metabolites that structurally and functionally mimic the mammalian sex hormone estrogen (Saviranta et al. 2008; Hashem and Soltan 2016). While phytoestrogens may have certain benefits to livestock agriculture, e.g., increasing growth, improving immunity, reducing trace gas emissions (Harlow et al. 2020; Jacobs et al. 2024), the overconsumption of phytoestrogen-rich legumes by livestock may negatively affect their reproductive performance (Bennetts et al. 1946; Thain 1965; Kallela et al. 1987; Hashem and Soltan 2016). Further, phytoestrogens consumed by lactating animals can be transferred to their milk in the form of equol, which is a potent phytoestrogenic metabolite derived from formononetin and daidzein by microorganisms present in the rumen (Batterham et al. 1965; Njåstad et al. 2014). The consumption of milk rich in equol could have health implications for livestock offspring and other individuals who consume milk (Gierus et al. 2012; Mayo et al. 2019; Dewulf et al. 2023).

The specific types and concentrations of phytoestrogen compounds vary among legume species. For instance, red clover (*Trifolium pratense* L.) can have relatively high levels of formononetin and biochanin A, while alfalfa (*Medicago sativa* L.) accumulates mostly genistein, coumestrol and kaempferol (Sivesind and Seguin 2005; Mustonen et al. 2018; Tucak et al. 2020). Even under controlled ambient conditions, baseline phytoestrogen concentrations can be as high as 5 mg g⁻¹ of dry matter in red clover and exceedingly low or undetectable in other legume species, such as kura clover (*T. ambiguum* L.) and forage cowpea (*Vigna unguiculata* L.) (Mandal et al. 2024, 2025). Moreover, phytoestrogen concentrations often vary among different cultivars of the same species (Sivesind and Seguin 2005; Papadopoulos et al. 2006). Consequently, variation in phytoestrogen concentrations across species and cultivars has important implications for the selection of forage legume species in relation to livestock reproductive health.

One of the major roles phytoestrogens are thought to play in plants is defense against biotic stressors (Trush and Pal'ove-Balang 2023). For example, research has shown that fungal pathogens can trigger the accumulation of phytoestrogens in red clover, alfalfa, and soybean (*Glycine max* L.) (Debnam and Smith 1976; Jasiński et al. 2009; Li et al. 2018, 2021). Similarly, aphid damage has been shown to increase coumestrol concentrations in alfalfa in some studies (Kain and Biggs 1980) but not others (Fields et al. 2018). Additionally, the content of formononetin in red clover was found to deter the clover root weevil (*Sitona lepidus* Gyll.) in a feeding trial, possibly acting as a chemical defense against

this herbivorous insect (Walling 2000; Gerard et al. 2005). However, despite compelling arguments that phytoestrogen production may also play additional defensive roles, including modulating the fertility of potential herbivores (Hughes 1988), relatively few studies have explicitly examined how insect damage to plant tissues may influence phytoestrogen concentrations, particularly in *Trifolium* species.

Understanding how insect damage may impact phytoestrogen responses in potentially phytoestrogenic perennial forage legumes is important for several reasons. First, perennial legumes, especially *Trifolium* species, are commonly used in temperate climates because of their adaptability and diverse uses as hay, silage, and grazed forage (Evers 2011). Second, warming atmospheric temperatures associated with climate change are likely to drive increases in insect pest abundance and damage in many temperate growing regions, and particularly the northeastern United States (Wolfe et al. 2008; Harvey et al. 2023). Lastly, perennial forage crops often receive minimal insect protection, as the primary focus of insect pest management is typically on protecting food crops. In pasturelands, factors such as plant species, grazing severity and frequency, and other abiotic and biotic conditions influence the abundance, duration, and dynamics of herbivorous insects and their natural enemies (Ritchie 2000; Dumont et al. 2009). As a result, the duration, severity, and scale of insect pest outbreaks and associated herbivory can vary widely (Kordan et al. 2018; Goldson et al. 2020). Understanding how these factors influence phytoestrogen concentrations in agronomically important forage legumes could help farmers better manage consumption of phytoestrogens by their livestock.

The objective of this study was to determine if insect herbivory influences phytoestrogen concentrations in three commonly grown temperate perennial forage legumes, red clover, white clover (*T. repens* L.), and kura clover. We chose these species because previous research has shown that they tend to have high, moderate, and low baseline phytoestrogen concentrations, respectively (Andersen et al. 2009; Mandal et al. 2024).

Materials and methods

Experimental setup and sampling

We conducted two experiments at the Macfarlane Research Greenhouse Facility at the University of New Hampshire in Durham, New Hampshire, United States. The first experiment (Experiment I) was conducted from October 28, 2021 to January 03, 2022 to investigate the effect of short-term (7 days) insect herbivory on phytoestrogen concentrations in red clover 'Freedom!' and kura clover 'VNS'. Based on

the results of Experiment I, we modified and expanded the treatments in the second experiment. In Experiment II, we incorporated another red clover cultivar, ‘Ruby Red’, substituted kura clover with white clover (‘Alice’), and extended the treatment duration to better understand longer-term effects of herbivory on phytoestrogen responses. Experiment II was conducted from March 1 to May 6, 2023. In both Experiments, mesh insect rearing cages (RestCloud®; 39.8 × 39.8 × 59.9 cm) were used to limit insect movement and protect plants from other insects in the greenhouse. The same rearing cages were also used to maintain similar experimental conditions in the control treatments, especially light availability. Seeds of all three clover species used in these experiments were purchased from a commercial supplier, Albert Lea Seed (Albert Lea, MN, USA).

At the end of the treatment duration of 7 days (Experiment I) and 18 days (Experiment II), we measured plant physiological responses, total phenolics, and phytoestrogen concentrations. In addition, to determine the damaged leaf area, all leaves from two randomly selected plants from each insect herbivory experimental unit were collected and scanned, and the images were analyzed with ImageJ software (version 1.54d). The plant samples, including leaves, stems, and flower tissue, were collected to fill a 50 mL conical centrifuge tube. The tubes were immediately placed on ice, flash-frozen in liquid N, and stored at −80 °C until biochemical analyses.

Experiment I

Clover species were grown in a completely randomized design with four replications. Plants were either subjected to insect herbivory (“herbivory”) or insects were excluded (“control”). Each species-treatment replication was an experimental unit (i.e., a single pot). Clover seeds were purchased from commercial seed companies and sown in 1.9 L pots (14.9 cm diameter × 12.9 cm height) in Promix BX growing media. After germination, plants were fertigated with 150 ppm N. Day length was set at 16 h with supplemental lighting to maintain a minimum daytime light intensity of 350 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The temperature and relative humidity were 21 °C and 60% during the day and 18 °C and 70% at night, respectively. A laboratory reared population of first instar larvae of corn earworm, *Helioverpa zea* (Boddie) were acquired from Benzon Research Inc. (Carlisle, PA, United States) and reared under the same greenhouse photoperiod with a standard diet until the third instar. Eight weeks after seeding, six third-instar larvae were released onto each plant designated for the ‘herbivory’ treatment and allowed to feed for 7 days.

Experiment II

Seeds of each of the three cultivars were sown in 3.8 L pots (17.0 cm diameter × 17.5 cm height) in Promix-BX potting media. Due to slower growth, white clover was sown two weeks earlier than red clover, so the two species would be at approximately the same developmental stage when treatments were applied. Once plants were at the early vegetative stage, pots were randomly assigned to one of four treatments: (i) insect herbivory damage for 18 days, (ii) insect herbivory damage for 9 days, followed by 9 days of recovery, (iii) mechanical injury, without insect herbivory, and (iv) a control, with no mechanical injury or insect herbivory. The experimental unit was one pot per treatment combination with four replications in a randomized complete block design.

Plants were grown in the greenhouse with 14 h light and 10 h dark conditions, 23 °C day and 18 °C night temperatures, and relative humidities of 60% (day) and 70% (night). Ten first-instar corn earworm larvae were released after five and seven weeks of seeding red clover and white clover, respectively, and allowed to feed for 9 or 18 days, according to experimental treatment. The mechanical injury treatment involved gradually damaging leaves with a hole punch, starting from the date of insect release and lasting for 18 days, to remove approximately 20–25% of the total leaf area. Additionally, at the end of the experiment, we collected damaged and undamaged plant tissue samples from plants subjected to the 18-day insect herbivory treatment to determine whether phytoestrogen concentrations varied between the two types of plant tissues. Due to limited resources, we were unable to assess within-plant variation for other three treatments.

Physiological parameters

To characterize plant physiological responses to leaf damage, we measured stomatal conductance, net photosynthetic rate, transpiration rate, substomatal CO₂ concentration, water use efficiency, and vapor pressure deficit with a portable photosynthesis system (CIRAS-3; PP Systems, Amesbury, MA, United States). A leaf cuvette (7 × 25 mm) with 390 $\mu\text{mol mol}^{-1}$ CO₂ reference, 250 cc min^{−1} cuvette flow, and a 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ LED light source was used. Measurements were taken from three randomly selected fully expanded leaves per experimental unit, within a window of ±2 h around solar noon (11:00–15:00). The chlorophyll content was measured utilizing a portable SPAD (SPAD 502, Konika Minolta Inc. Tokyo, Japan) meter from three fully expanded leaves per experimental unit.

Total phenolic content

Total phenolic content from the plant tissue sample was measured using the methodology outlined by Ainsworth and Gillespie (2007). Approximately 20 mg of freeze-dried ground plant material was added to 15 mL centrifuge tubes and mixed with 2 mL of ice-cold 95% methanol. Samples were vortexed and left in the dark at ambient temperature for 48 h with intermittent vortexing throughout this period. Next, samples were centrifuged at 2,350 g for 5 min at room temperature and clear supernatant was transferred to a new 2 mL microtube. The supernatant from each sample (100 μ L), gallic acid standard, or 95% methanol (serving as a blank) were pipetted in triplicate 2 mL microtubes. After this, 200 μ L of a 10% Folin-Ciocalteu reagent was added to each microtube, followed by the addition of 800 μ L of a 700 mM Na_2CO_3 solution. The microtubes were mixed well and incubated at room temperature for 2 h. After vortexing, 200 μ L sample from the test tube was transferred into a clear 96-well plate, and the absorbance was recorded at a wavelength of 765 nm in a microplate reader (Molecular Devices Spectra MAX 190, Sunnyvale, CA, United States). A standard curve was constructed by plotting the absorbance values of gallic acid standards ranging from 0 to 1.5 mM. Total phenolic content was quantified as gallic acid equivalents (GAE) per gram of plant tissue dry weight using the regression equation from the gallic acid calibration curve.

Phytoestrogen analysis

Phytoestrogens were measured from plant samples collected from each experimental unit. Phytoestrogens, specifically formononetin, biochanin A, daidzein, genistein, glycitein, prunetin, and coumestrol, were measured using high-performance liquid chromatography (HPLC) according to Payette et al. (2021). Plant tissue samples were freeze-dried and then ground into a powder. Approximately 100 mg were transferred into 15 mL centrifuge tubes, followed by the addition of 4750 μ L 80% aqueous methanol and 250 μ L internal standard flavone (400 ng μL^{-1}). This mixture was vortexed and then subjected to sonication for 10 min at room temperature. After sonication, samples were shaken in a multitube vortexer for 2 h and left soaking overnight. Samples were spun down to sediment biomass and the supernatant (1.5 mL) was filtered through a 0.45 μm PVDF syringe filter into a 2 mL HPLC vial and refrigerated at 4 $^{\circ}\text{C}$ until subjected to HPLC analysis within the next 24 h. Phytoestrogens were identified and quantified using an HPLC system (Agilent Technology 1100 Series, Palo Alto, CA, United States) equipped with a diode array detector. Chromatographic separation was performed at a 0.3 mL min^{-1} flow rate using an Atlantis T3 C_{18} column (150 $\times$ 2.1, 3 μm ;

Waters Corp., Milford, MA, United States) maintained at 40 $^{\circ}\text{C}$. The mobile phase was constituted of solvent A (H_2O with 0.1% formic acid) and solvent B (CH_3CN). Samples were separated by a gradient elution of 15% to 50% CH_3CN over the first 35 min, followed by a sharp increase to 85% in the last 5 min of the run. To quantify the phytoestrogens, the detector was set at a wavelength of 260 nm. Phytoestrogen compounds were identified in the test samples by matching their retention times and UV–visible spectra against the reference of formononetin, biochanin A, daidzein, genistein, glycitein, coumestrol, and prunetin standards. The calibration curves were prepared using these standards to quantify the phytoestrogen concentration of the test samples. The final concentration of each phytoestrogen was adjusted and expressed based on the dry matter (DM) weight of the samples.

Data analysis

Statistical analyses were performed on data from both experiments using R Studio version 2024.04.0. The treatment effect on plant physiological and biochemical response variables was assessed using analysis of variance (ANOVA) with partial (type II) sums of squares. Tukey's HSD test ($\alpha=0.05$) was used to determine significant treatment differences. In Experiment I, the statistical model included treatment and species as fixed effects. In Experiment II, the model included block, cultivar (red clover only), and treatment, all as fixed effects. To compare phytoestrogen levels within insect damaged and undamaged plant tissues of plants exposed to 18-days of herbivory, the model included block, leaf tissue (damaged vs. undamaged) and cultivar (red clover only). These within-plant comparisons should be interpreted with caution given their lack of independence. Homogeneity of variances and normal distribution of residuals were analyzed using residual plots and the Levene ('car' R package) and Shapiro–Wilk tests, respectively. Variables that failed to meet variance and distributional assumptions were log transformed. After log transformation, several variables measured in Experiment I failed to meet variance and distributional assumptions; these included net photosynthesis, water use efficiency, total phytoestrogen concentration, and concentrations of formononetin, biochanin A, genistein, and prunetin. For these variables, the nonparametric Kruskal–Wallis test was used. In instances where a significant main effect of cultivars and a significant interaction between treatments and cultivars were detected, Tukey's HSD test ($\alpha=0.05$) was used to determine significant differences among treatments, with results presented separately for each cultivar. For all other variables, when the ANOVA indicated significant treatment effects, Dunnett's test ("multcomp"

package) was applied to compare each treatment with the control.

Results

Experiment I

Physiological responses and total phenolic content

Leaf area damaged by insect herbivores was calculated after seven days of insect herbivory. Corn earworm consumed 8.0% and 13.2% of the leaf area in red clover and kura clover, respectively. The insect herbivory treatment had no effect on physiological responses (Supplementary Table S1) in either species, except for the SPAD value (Fig. 1).

The leaf SPAD value was influenced by both insect herbivory and legume species (treatment: $p < 0.05$, species: $p < 0.05$, treatment $\times$ species: $p = 0.93$). Kura clover had greater leaf SPAD values than red clover (Fig. 1A), and compared to the control, leaf SPAD values in both clover species were greater in the insect herbivory treatment (Fig. 1B). The total phenolic content was unaffected by insect herbivory or clover species (treatment: $p = 0.14$, species: $p = 0.19$, treatment $\times$ species: $p = 0.10$, Supplementary Table S1).

Phytoestrogen concentration

Formononetin, biochanin A, genistein, and prunetin were detected, whereas the levels of other targeted phytoestrogens

(daidzein and glycitein) were below the detection level. A representative chromatogram of red clover ‘Freedom!’ and kura clover ‘VNS’ are shown in Supplementary Fig. S1A and D, respectively. The total phytoestrogen concentration was 27 times greater in red clover than in kura clover ($p < 0.05$). The effect of insect herbivory on the total phytoestrogen concentration depended on clover species (treatment $\times$ species: $p < 0.01$). In red clover, insect herbivory increased total phytoestrogen concentration by 112% (6.8 mg g^{-1} of DM) compared to undamaged red clover (3.2 mg g^{-1} of DM; $p < 0.05$), whereas in kura clover total phytoestrogen concentration remained extremely low regardless of insect herbivory treatment (Fig. 2). The increase in total phytoestrogen concentration observed in insect-damaged red clover was driven primarily by increases in the individual phytoestrogen compounds formononetin (treatment $\times$ species: $p < 0.01$) and biochanin A (treatment $\times$ species: $p < 0.05$) (Fig. 2 B and C, respectively). The concentration of genistein was also greater in damaged compared to control red clover (treatment $\times$ species: $p < 0.05$; Fig. 2D), while prunetin concentration did not differ between treatments (treatment $\times$ species: $p > 0.05$; Fig. 2E). In kura clover, insect herbivory did not influence concentrations of any individual phytoestrogen compound (treatment $\times$ species: $p > 0.05$; Fig. 2).

Experiment II

Physiological responses

Following 18 days of insect herbivory, damaged leaf area was 31.0%, 28.4%, and 30.3% for ‘Freedom!’, ‘Ruby Red’, and ‘Alice’, respectively. Overall, physiological responses differed among clover species and cultivars. Substomatal CO_2 concentration, stomatal conductance, net photosynthesis, and transpiration rate were greater, while water use efficiency and vapor pressure deficit were lower in white clover compared to red clover (Table 1). Among the physiological responses we measured, only the transpiration rate differed between the two red clover cultivars. However, insect herbivory treatments did not significantly affect these parameters, except for a marginal increase in net photosynthesis ($p = 0.05$; Table 1).

The effect of insect herbivory on leaf SPAD value depended on clover species/cultivars (treatment $\times$ cultivar: $p < 0.05$). Within each species/cultivar, mean leaf SPAD values were greater in the 18-day insect herbivory compared to other treatments (Fig. 3A). SPAD values did not differ between the two red clover cultivars. In absence of insect damage, mechanical damage through leaf punching did not induce changes in SPAD value compared to the control (Fig. 3A). There were no significant differences in physiological variables between the 18-day continuous herbivory

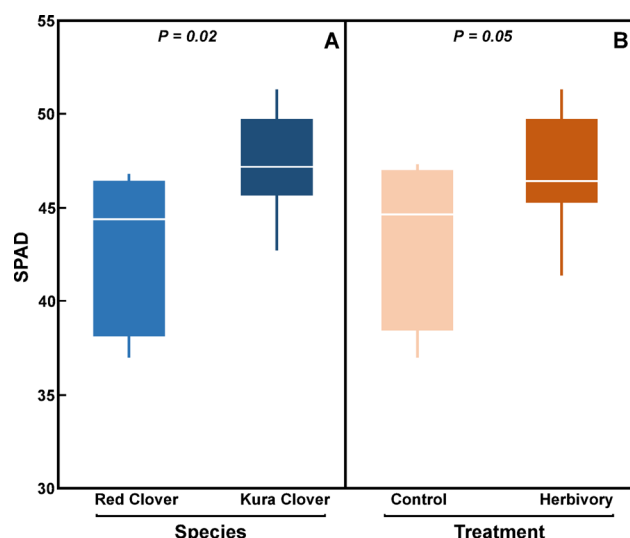


Fig. 1 Boxplots showing leaf SPAD values based on species (A) and herbivory treatment (B) in response to 7 days of insect herbivore damage in Experiment I. The horizontal line inside the boxplot indicates the median and the box represents the interquartile range. Whiskers extending from the box represent the minimum and maximum value in the data; $n = 8$

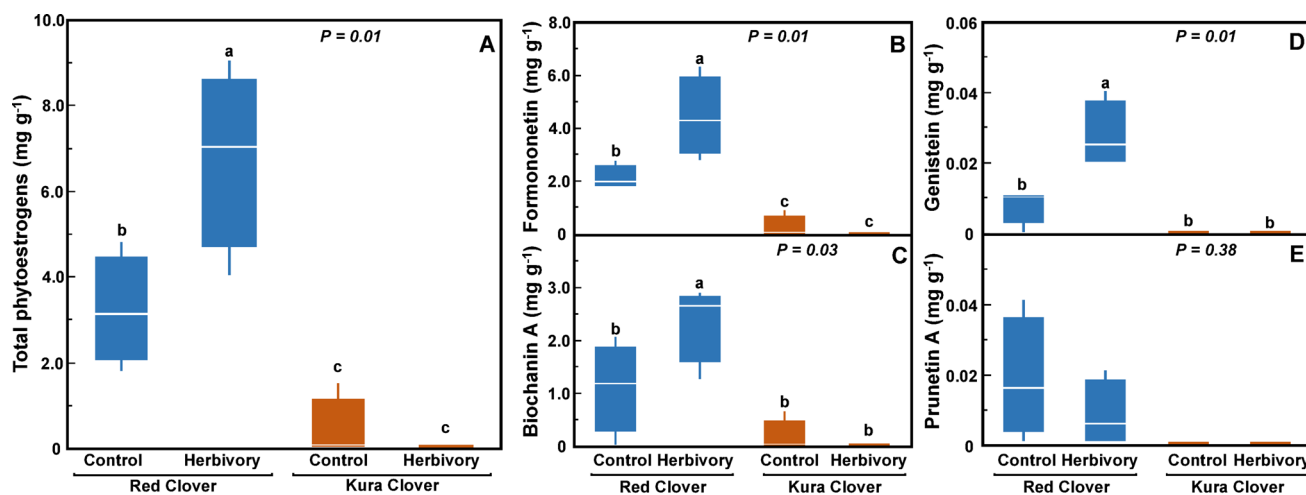


Fig. 2 Effects of 7 days of insect herbivore damage on concentrations of total phytoestrogens (A), and the individual phytoestrogen compounds formononetin (B), biochanin A (C), genistein (D), and prunetin (E) in red clover and kura clover (mg g^{-1} of DM) in Experiment I. The horizontal line inside the boxplot indicates the median and the box

represents the interquartile range. The vertical lines extending from the box represent the minimum and maximum value in the data; $n=4$. Treatments sharing the same letter are not significantly different ($\alpha=0.05$) as determined by Tukey's HSD test

Table 1 Results of ANOVA for physiological responses (treatment means $\pm$ (1) SE, $n=4$) of red clover cultivars 'Freedom!' and 'Ruby Red' and white clover 'Alice' to 9 and 18 days of insect herbivore damage in Experiment II

Variables ^a	Treatments				Species/Cultivars			Treatments (T)	Species (S)/ Cultivar(C) ^b	S/C $\times$ T
	Control	Insect herbivory		Mechanical damage	Freedom!	Ruby Red	Alice			
		9-day	18-day							
Ci	226.3 $\pm$ 17.2	246.1 $\pm$ 11.9	237.3 $\pm$ 11.3	229.3 $\pm$ 15.5	231.1 $\pm$ 5.03	228.7 $\pm$ 3.60	262.4 $\pm$ 2.36	$p=0.64$	$p<0.01$	$p=0.90$
Gs	608.7 $\pm$ 124	724.3 $\pm$ 101.8	634.3 $\pm$ 83.8	663.1 $\pm$ 98.2	457.4 $\pm$ 21.9	623.6 $\pm$ 22.8	891.7 $\pm$ 28.5	$p=0.62$	$p<0.01$	$p=0.85$
A	23.8 $\pm$ 1.0	26.1 $\pm$ 1.2	26.8 $\pm$ 0.8	26.5 $\pm$ 1.2	24.3 $\pm$ 0.41	26.0 $\pm$ 0.27	27.1 $\pm$ 0.21	$p=0.05$	$p<0.05$	$p=0.71$
E	12.2 $\pm$ 1.3	13.8 $\pm$ 1.1	13.0 $\pm$ 1.0	13.1 $\pm$ 1.3	10.6 $\pm$ 0.33	13.0 $\pm$ 0.31	15.4 $\pm$ 0.26	$p=0.54$	$p<0.01$	$p=0.87$
VPD	3.0 $\pm$ 0.2	2.8 $\pm$ 0.1	2.8 $\pm$ 0.1	2.8 $\pm$ 0.1	3.1 $\pm$ 0.04 a	3.0 $\pm$ 0.03 a	2.6 $\pm$ 0.03 b	$p=0.27$	$p<0.01$	$p=0.95$
WUE	2.3 $\pm$ 0.2	2.1 $\pm$ 0.2	2.3 $\pm$ 0.2	2.4 $\pm$ 0.3	2.6 $\pm$ 0.07 a	2.3 $\pm$ 0.05 ab	1.9 $\pm$ 0.04 b	$p=0.72$	$p<0.05$	$p=0.88$

^aCi, sub-stomatal CO_2 concentration ($\mu\text{mol mol}^{-1}$); gs, stomatal conductance ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$); A, net photosynthetic rate ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$); E, transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$); VPD, vapor pressure deficit (kPa); WUE, water use efficiency ($\text{mmol CO}_2 \text{mol H}_2\text{O}$)

^bWhere ANOVA test indicated a significant difference ($p<0.05$) for species/cultivars, means were separated by Tukey's HSD test; treatment sharing the same letter are not significantly different ($\alpha=0.05$)

treatment and the 9-day herbivory followed by 9-day recovery treatment.

The total phenolic content differed among clover species/cultivars and treatments (treatment: $p<0.05$; species/cultivars: $p<0.01$; treatment $\times$ species/cultivars: $p=0.89$). The red clover cultivars had lower total phenolic content compared to white clover 'Alice' (Fig. 3B). In all three cultivars, total phenolic concentration was higher after 18 days of continuous insect herbivory compared to when herbivory occurred for 9 days followed by a 9-day recovery period without herbivory (Fig. 3C).

Phytoestrogen concentration

Across the treatments, total phytoestrogen concentrations in red clover (3.62 mg g^{-1} of DM) were approximately 86 times greater than in white clover (0.042 mg g^{-1} of DM). Representative chromatograms of red clover and white clover are shown in Supplementary Fig. S1A–C. Hence, statistical analyses were conducted independently for each species to ensure that the orders-of-magnitude difference between species did not mask important treatment effects. In red clover, the total phytoestrogen concentration did not differ between the two cultivars (cultivar: $p=0.42$, cultivar $\times$ treatment

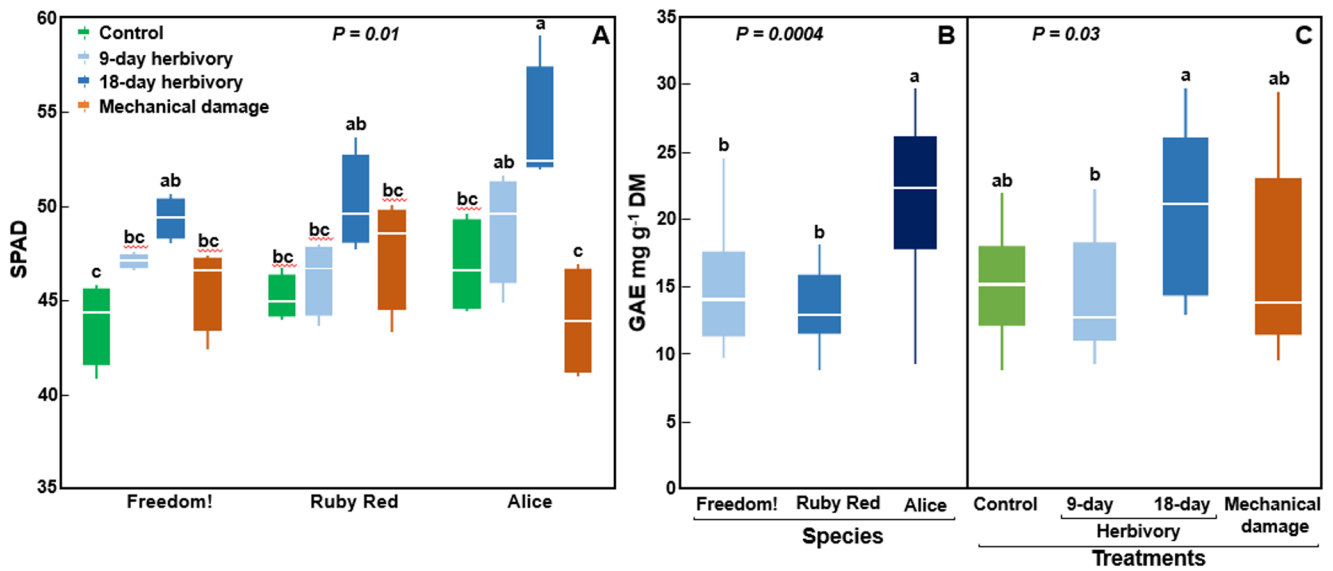


Fig. 3 Effects of experimental treatments on leaf SPAD value (A) and effects of species/cultivar (B) and insect herbivory (C) on total phenolic content in Experiment II. The horizontal line inside the box indicates the median and the box represents the interquartile range.

The vertical lines extending from the box represent the minimum and maximum value in the data; $n=4$ (A), 16 (B) and 12 (C). Treatments sharing the same letter are not significantly different ($\alpha=0.05$) as determined by Tukey's HSD test

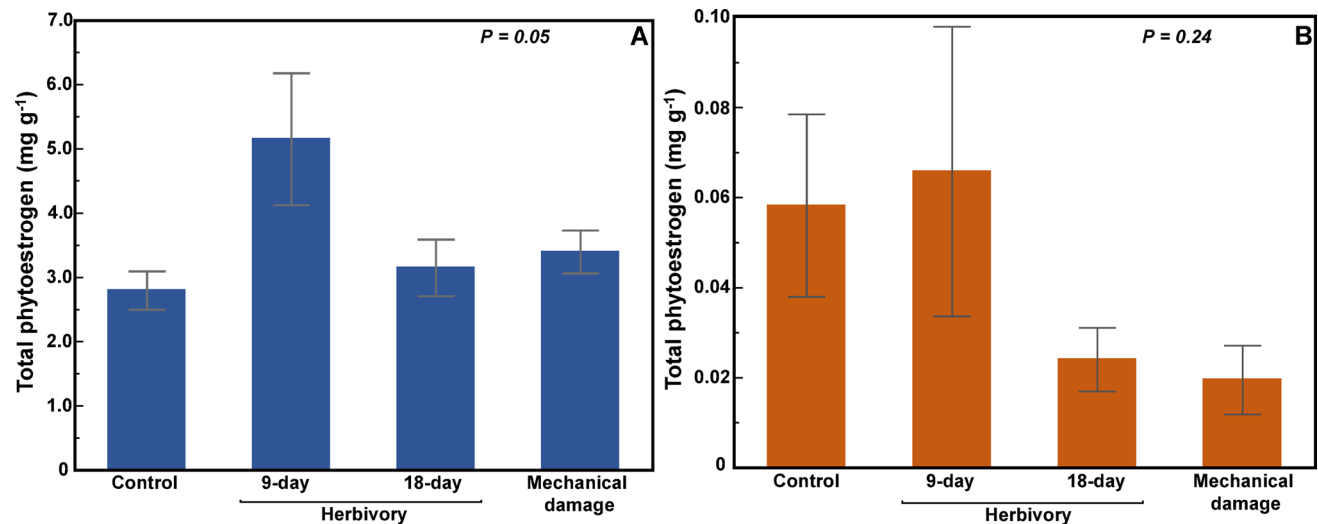


Fig. 4 Effects of insect herbivory on total phytoestrogen concentration (mg g⁻¹ of DM) in two red clover cultivars (A) and white clover (B) in Experiment II. Error bars are $\pm(1)$ SE of the mean; $n=8$ (A) and 4 (B)

$p=0.99$) but did show evidence of a possible treatment effect (treatment: $p=0.05$). Specifically, mean total phytoestrogen concentration in red clover exposed to 9-days of insect herbivory followed by a 9-day recovery period was 84% higher compared to the control; however, this difference was not significant (Dunnett's test, $p>0.05$) (Fig. 4A). In contrast, mean total phytoestrogen concentration in red clover was similar to the control when herbivores continuously damaged plants for 18 days and when mechanically damaged using a leaf punch, respectively. Total phytoestrogen concentration in white clover did not differ in any treatment (treatment: $p=0.25$, Fig. 4B).

Individual phytoestrogen compounds in red clover responded differently to treatments. Formononetin concentration did not differ between the two red clover cultivars (cultivar: $p=0.28$, cultivar $\times$ treatment $p=0.96$) but did show evidence of a possible treatment effect (treatment: $p=0.05$). Specifically, mean formononetin concentration in red clover in the 9-day herbivory followed by recovery period treatment was 76.6% higher than in the control (Fig. 5A). Biochanin A, genistein, daidzein, and prunetin concentration did not differ between the two cultivars and were not influenced by the treatments (treatment: $p>0.05$, cultivar: $p>0.05$, cultivar $\times$ treatment: $p>0.05$; Fig. 5B,

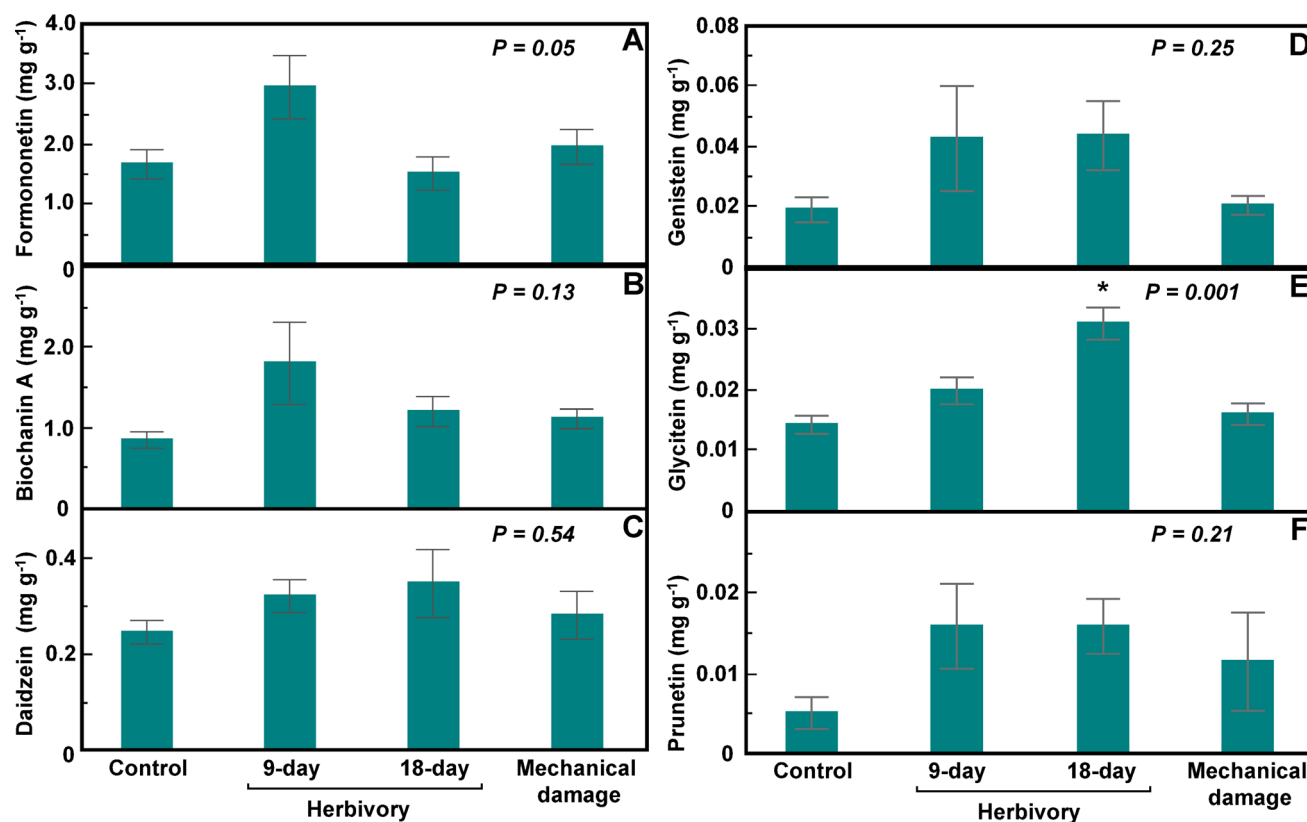


Fig. 5 Effects of insect herbivory on the individual phytoestrogens (mg g⁻¹ of DM) formononetin (A), biochanin A (B), daidzein (C), genistein (D), glycitein (E), and prunetin (F) in two cultivars of red clover in

Experiment II. Error bars are $\pm(1)$ SE of the mean; $n=8$. Treatments with an asterisk (*) are statistically different from the control treatment according to Dunnett's test ($\alpha=0.05$)

C, D, F). Glycitein concentration was higher in the 18-day insect herbivory treatment compared to other treatments (treatment: $p<0.01$; Fig. 5E), but did not differ between red clover cultivars (cultivar: $p=0.75$, cultivar $\times$ treatment: $p=0.34$). In contrast, individual phytoestrogen compounds in white clover did not differ (treatment: $p>0.05$) regardless of treatment (Supplementary Table S2).

In addition to our whole-plant analyses, in the 18-day herbivory treatments we also investigated if phytoestrogen concentrations varied between damaged and undamaged tissues within the same plant. In red clover, the undamaged tissues accumulated greater total phytoestrogens compared to the insect-damaged tissues ($p<0.01$, Fig. 6A). In white clover, damaged and undamaged tissues did not differ in phytoestrogen concentration ($p>0.05$; Fig. 6B).

Discussion

In nature, plants are exposed to a variety of biotic stresses, including herbivorous insects, and respond by producing various secondary metabolites to defend themselves (Bennett and Wallsgrave 1994; Simmonds 2003; Jha and Mohamed 2022). We found that insect herbivory can

increase the production of phytoestrogens in red clover; however, the magnitude of this effect at the whole-plant level appears to depend on the duration of herbivory. Considering both experiments together, whole-plant phytoestrogen concentrations were greatest immediately following seven days of herbivory and were nearly just as high after nine days of herbivory, despite being measured a full nine days after herbivores were removed from the plant. In contrast, total phytoestrogen concentrations did not differ from the control when red clover plants were exposed to 18 days of continuous herbivory damage.

Our study has several limitations that constrain our ability to determine conclusively the mechanisms underpinning the results we observed. One limitation is that in both experiments our phytoestrogen measurements were restricted to a single time point. Because of this, we do not know if or how phytoestrogen concentrations may have changed from the time of initiation to the cessation of herbivory in either experiment or over the course of the nine-day recovery period following the initial nine days of insect herbivory in experiment II. A second limitation is the fact that supplemental measurements of phytoestrogens on insect-damaged and undamaged red clover tissues occurred only on plants in the 18-day herbivory treatment. Hence, we do not know

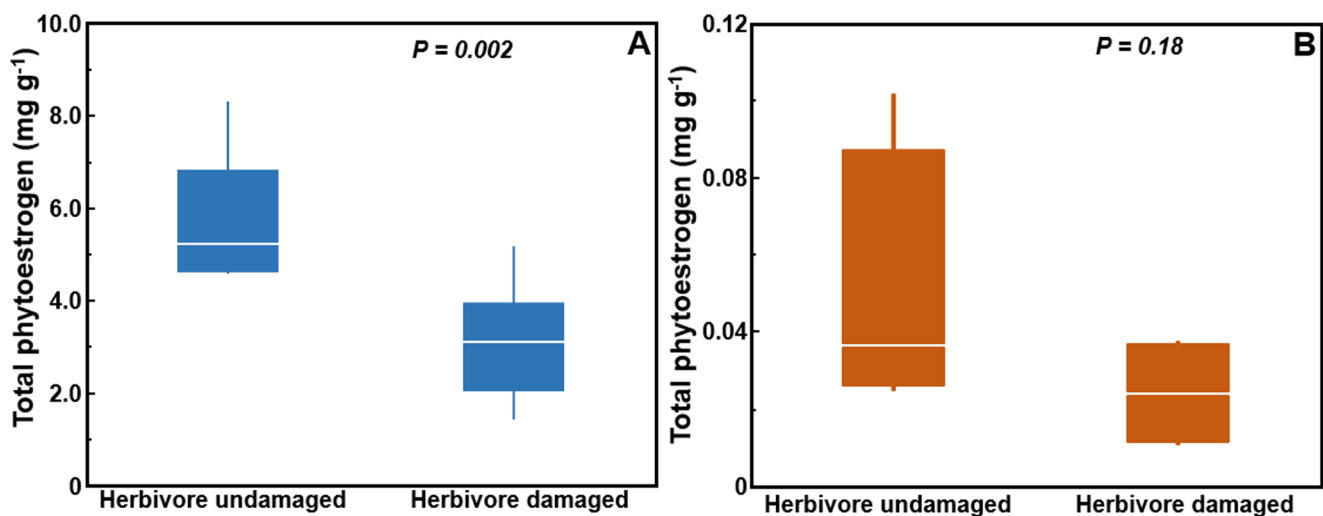


Fig. 6 Total phytoestrogen concentration (mg g⁻¹ of DM) in damaged and undamaged tissues sampled on individuals of red clover (**A**) and white clover (**B**) following 18-days of insect herbivory in Experiment II. The horizontal line inside the boxplot indicates the median and the

box represents the interquartile range. The vertical lines extending from the box represent the minimum and maximum value in the data; $n=8$ (**A**) and 4 (**B**)

if localized accumulation of phytoestrogens (in undamaged tissues) was specific only to this treatment or occurred in other treatments. It is important to note, however, this does not limit the applied relevance of our findings, as ruminant livestock would consume both damaged and undamaged tissues. These caveats aside, we offer several potential explanations for our observations.

Our observation in red clover that phytoestrogen concentrations were highly elevated following 7 and 9 days of herbivory, even after a 9-day recovery period, is suggestive of a priming effect. Plants often exhibit ‘priming’ (Hilker and Schmülling 2019), where the production of defensive compounds like phytoestrogens remains elevated after stress. The priming process readies plants for potential reinfestation by herbivores, minimizing their lag time to respond biochemically to future attacks (Frost et al. 2008; Conrath et al. 2015). The length of time a plant stays primed likely depends on multiple factors, including herbivore attack frequency and intensity, plant species, and the priming strategy. For example, herbivore-induced plant volatiles (HIPVs) triggered defense responses lasting up to 2 days in lima bean (*Phaseolus lunatus* L.) and up to 10 days in maize (Choh and Takabayashi 2007; Ali et al. 2013), while some cultivars of tomato do not exhibit priming responses (Zhang et al. 2020).

Relatedly, isoflavone synthesis may be triggered by systemic signaling pathways (e.g., jasmonic acid or salicylic acid) that, once activated, may sustain isoflavone production for some time, even after the removal of insect herbivory (Durrant and Dong 2004; Algar et al. 2014). Such systemic responses are consistent with previous findings in other legumes. For example, Algar et al. (2014) demonstrated

that isoflavones in soybean play a role in systemic resistance to bacterial pathogens, and that this response involves coordinated signaling pathways such as salicylic acid and jasmonic acid. How long phytoestrogen concentrations can remain elevated in red clover following a short-term (e.g., 7–9 days) insect herbivory event requires additional investigation.

The fact that whole-plant phytoestrogen concentrations were elevated when measured immediately following 7 days of herbivory (Experiment I) but were not elevated when measured immediately following 18 days of herbivory (Experiment II) suggests there may be an energetic cost associated with priming. Additionally, our observation that phytoestrogens were elevated in undamaged tissues but not damaged tissues of insect-defoliated plants following 18 days of herbivory helps explain why total phytoestrogen concentrations appeared relatively unaltered in this treatment compared to the control. It may also support the notion that energetic costs incurred over longer periods of insect herbivory (i.e., >7–9 days) can alter the production and transport of phytoestrogens and/or priming. There could be several explanations for these patterns. First, previous research has shown that secondary metabolite responses, including the production of isoflavones, are often localized and vary between species and environmental conditions (Erb et al. 2012). This localized response may occur when the cost of systemic defense outweighs its benefits or the plant lacks the vascular or signaling capacity to mount a whole-plant defense. Second, prolonged insect herbivory may induce senescence or chronic stress responses in plants, where the focus shifts from the production of phytoalexins that affect the survival of insects (Karban and Myers 1989).

Studies have shown that phytoestrogens like formononetin, daidzein, and genistein, whether provided in no-choice feeding trials or consumed via legume tissue herbivory, suppress feeding and insect weight gain, indicative of a defense strategy (Goławska and Łukasik 2012; Quiroz et al. 2017). Third, insect herbivory can lead to hormonal crosstalk where other signaling pathways (e.g., abscisic acid or ethylene) become dominant, potentially suppressing jasmonic acid-mediated defenses (Verhage et al. 2010). Finally, insect herbivory may lead to resource depletion, forcing the plant to prioritize growth and tissue repair over continued defense metabolite production (Herms and Mattson 1992). Our observation that insect-damaged plants exhibited greater leaf SPAD values compared to undamaged plants suggests that plants compensated for a decrease in photosynthetic capacity due to a loss in leaf area by increasing the chlorophyll content of the remaining leaf tissue, as has been observed in other plants like goldenrod (*Solidago* species; Meyer 1998).

Our observation in Experiment II that phytoestrogen concentration did not differ between the mechanically damaged and undamaged control plant suggests that insect-derived factors such as saliva and oral secretions may stimulate phytoestrogen accumulation. However, biochemical responses of plants to insect herbivores, including phytoestrogen accumulation, can be species-specific and are influenced by multiple factors, including the nature, severity, and duration of the damage (Zhou et al. 2015; Ramarosan et al. 2022). The feeding habits of specific insect herbivores may also influence a plant's biochemical response; however, few studies have explicitly examined this. In previous work, it has been demonstrated that leaf damage from both piercing-sucking (stink bugs, *Nezara viridula* L.) and chewing (cotton leafworm, *Spodoptera litura* F.) insects can increase isoflavone concentrations in soybean leaves and seeds (Piubelli et al. 2003; Murakami et al. 2014; Hohenstein et al. 2019; Sabljic et al. 2020). Similarly, in alfalfa, feeding by the pea aphid (*Acyrtosiphon pisum* Harris), a piercing-sucking insect, increased coumestrol levels (Fields et al. 2018). In agreement with our results, Gerard et al. (2005) reported that red clover root damage by chewing insects influenced the phytoestrogen content in aboveground tissues. To the best of our knowledge, however, ours is the first study to evaluate the effects of chewing insects feeding on red clover leaf tissue. Whether red clover exhibits similar phytoestrogen responses with other chewing insect herbivores or species that feed via piercing-sucking requires additional research.

Results of this study revealed significant differences in phytoestrogen levels among the clover species we examined. Notably, phytoestrogen concentrations were 86 times greater in red clover compared to white clover, while concentrations in kura clover were often nondetectable, which is

in line with previous research (Saloniemi et al. 1993; Andersen et al. 2009; Sheaffer et al. 2018; Mandal et al. 2024). Collectively, these results highlight the substantial variation in phytoestrogen concentrations across different *Trifolium* species, reinforcing the importance of species and cultivar selection when managing potential phytoestrogen exposure in forage-based livestock systems, particularly where insect herbivory occurs regularly. Interestingly, across all treatments, the total phenolic content was greater in white clover compared to red clover, which contrasts with findings from some earlier studies (Koc et al. 2016; Tava et al. 2019).

Our findings that formononetin and biochanin A were the two major phytoestrogens in both red clover cultivars we examined, cumulatively comprising around 90% of the total phytoestrogens measured, are in line with previous research (Tsao et al. 2006; Mustonen et al. 2018; Popović et al. 2019). Formononetin itself has a greater estrogenicity than many other phytoestrogen compounds and is of particular concern because it can be converted into equol by microbiota in the rumens of ruminant species (Njåstad et al. 2014). Equol has greater estrogenic activity than formononetin (Mayo et al. 2019), and it can also be transferred to milk (Pfischer et al. 2008; Höjer et al. 2012). Equol in milk may have implications for human health (Magee 2011).

Previous research revealed that formononetin concentrations $> 3 \text{ mg g}^{-1}$ of DM can pose reproductive health problems in sheep; however, concentration levels that may adversely affect other animals are not well understood (Wyse et al. 2022). Some research suggests formononetin concentrations ranging from as low as 0.5 to 0.75 mg g^{-1} of DM may be associated with infertility in cattle (Mostrom and Evans 2011; Wyse et al. 2021). The concentration of formononetin in red clover in the control treatment across our two studies averaged 1.8 mg g^{-1} of DM. Our finding that short-duration insect herbivory damage (7–9 days) led to formononetin concentrations of 3.4 mg g^{-1} to 4.4 mg g^{-1} of DM indicates that insect herbivory resulted in red clover forage with potential to negatively impact reproductive health risk for some ruminant livestock.

Conclusions

Our findings highlight that insect herbivory damage increases phytoestrogen concentrations in red clover, with the response depending on the duration of damage. Short-term herbivory (7–9 days) results in elevated phytoestrogen levels, potentially as part of a priming or systemic acquired resistance mechanism. In contrast, relatively prolonged periods of insect herbivory (18 days) result in elevated phytoestrogen levels localized in undamaged plant tissues, resulting in whole-plant concentrations that do not differ

from those of undamaged plants. These results raise new avenues for research and insights for management practices that can help farmers better balance pest management, grazing, and harvest decisions to minimize phytoestrogen exposure to ruminants.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11829-025-10206-5>.

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Data availability The datasets used and/or analyzed in this study are available upon reasonable request to the corresponding author.

Declarations

Conflict of interest The authors declare no competing interests.

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