Are the Phytoestrogens Genistein and Daidzein Anti-Herbivore Defenses? A Test Using the Gypsy Moth (*Lymantria dispar*)

David Nathan Karowe · Joshua Karl Radi

Abstract Phytoestrogens are compounds that have moderate estrogenic or anti-estrogenic activity toward mammals. Although genistein and daidzein, the main phytoestrogens of soybean, have been the subject of thousands of studies that address their benefit to human health, relatively little is known about their benefits to plants that produce them. It has been suggested that genistein and daidzein protect plants against arthropod herbivores, but direct tests of this hypothesis are rare. In this study, we evaluated the effect of genistein and daidzein on the survivorship, growth, and fecundity of the gypsy moth, a generalist insect herbivore that does not encounter phytoestrogens in its normal diet. We compared survivorship, egg-to-pupa growth rate, and 4th instar performance of gypsy moth caterpillars on artificial diets containing no phytoestrogen, genistein, daidzein, or a combination of genistein and daidzein. Our results indicate that genistein and daidzein do not decrease survivorship, growth, or fecundity of this insect herbivore. Therefore, it seems unlikely that the primary function of these compounds in aboveground plant tissues is anti-herbivore defense.

Key Words Genistein · Daidzein · Phytoestrogen · Gypsy moth · Larval performance · Anti-herbivore defense

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Introduction

Phytoestrogens are plant compounds that, because of their structurally similarity to estradiol, have moderate estrogenic or anti-estrogenic activity toward mammals. Phytoestrogens occur in over 300 species of plants, most notably legumes (Tham et al., 1998). Genistein and daidzein (Fig. 1), the primary phytoestrogen isoflavones of soybean, have been the subject of thousands of studies that address their beneficial effects on human health, including hormone-dependent breast and prostate cancers, osteoporosis, cardiovascular disease, immunity and inflammation, and reproduction and fertility in humans and other mammals (Cornwell et al., 2004; Xiao, 2008; Andres et al., 2009; Baber, 2010; Bhupathy et al., 2010).

Phytoestrogens have been proposed to confer three types of benefit to plants: facilitation of associations with below-ground mutualists including nitrogen-fixing bacteria and mycorrhizal fungi; protection against pathogens; and protection against herbivores. The first two are well-documented. Numerous studies have demonstrated the role of phytoestrogens in attracting and promoting colonization by mutualistic bacteria (Schultze and Kondorosi, 1998; Makoi and Ndakidemi, 2007; Bhattacharya et al., 2010) and fungi (Requena et al., 2007; Badri et al., 2009). It is also well established that the phytoalexin glyceollin, for which daidzein is a precursor, is induced by and protects plants against fungal pathogens (Lozovaya et al., 2007; Jasinski et al., 2009; Lygin et al., 2010). However, the efficacy of phytoestrogens as anti-herbivore compounds is far less clear.

Only a few studies have addressed the potential anti-herbivore benefits of phytoestrogen isoflavones, and most of these provide only indirect evidence. For instance, soybean extracts containing genistein and/or daidzein possess antifeedant or antibiotic activity toward mites
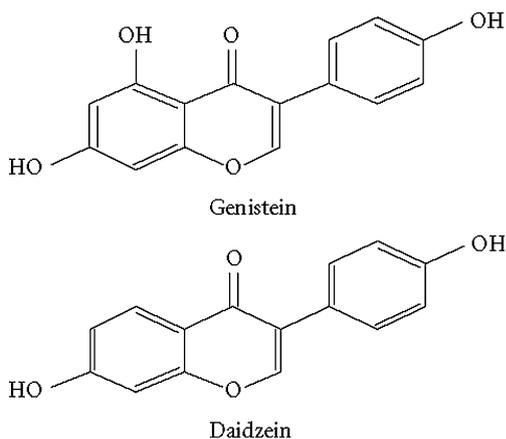
Fig. 1 Genistein and daidzein

(Wang et al., 1999), stink bugs (Piubelli et al., 2003), and lepidopterans (Hoffmann-Campo et al. 2001; Piubelli et al., 2005). However, as the authors note, these extracts contained many compounds, so it is not possible to unequivocally attribute deterrence or antibiosis to genistein and daidzein.

More convincing, but far less common, are controlled studies in which genistein and/or daidzein are applied to a neutral medium. Unfortunately, even in such controlled studies, evaluation of the anti-herbivore effects of genistein and daidzein is complicated by uncertainty about the ecological relevance of doses used (Sutherland et al., 1980; Sharma and Norris, 1991).

In a recent review of insect-soybean interactions, Parrott et al. (2008) called attention to "a notable lack of supportive research" into the role of phytoestrogens as deterrents to herbivory. We report here the results of a controlled study in which a generalist herbivore, the gypsy moth, was exposed to naturally-occurring levels of the two most abundant soybean phytoestrogens, genistein and daidzein.

Methods and Materials

The gypsy moth (*Lymantria dispar*) is a generalist herbivore that feeds on leaves of deciduous trees. Because gypsy moths do not feed on phytoestrogen-containing plants, we considered it to be particularly likely to be sensitive to genistein and/or daidzein.

Gypsy moth caterpillars were reared from hatching through the end of the final instar on gypsy moth artificial diet; both gypsy moth eggs and diet were generously provided by V. Mastro and C. Lokerson of the U.S.D.A. Plant Protection and Quarantine lab in Otis, MA, USA. Genistein and daidzein, the two major phytoestrogens in soybean (Liggins et al., 2000; Zhu et al., 2005), were purchased from LC Laboratories (Woburn, MA, USA). The

rearing diet contained no phytoestrogen (control), 0.84 mg/g dry weight genistein, 0.84 mg/g dry weight daidzein, or 0.42 mg/g dry weight of both genistein and daidzein. These levels were chosen to reflect the highest reported concentration of genistein in any aboveground plant tissue (soybeans) (Dixon, 2004), and are substantially higher than levels found in leaves (Mazur and Adlercreutz, 1998).

Effect of Phytoestrogens on Survivorship To determine whether genistein and/or daidzein affects gypsy moth survivorship, 10 newly hatched larvae from each of 28 full-sib families were placed together into each of four 25-ml polystyrene cups in an environmental chamber at 30°C with a 16:8 hL:D cycle (*N*=1120 larvae). Each cup contained one of the four diets (control, genistein, daidzein, or genistein+ daidzein). Larvae were given fresh diet every 3 d. Upon molting into the 3rd instar, the 10 larvae from each family were divided equally between two cups to ensure sufficient space for the insects.

The first two larvae from each family on each diet that molted into the 4th instar were removed and used as wet/dry standards for the nutritional index study described below, and the fifth larva was used to measure nutritional indices. The third, fourth, sixth, and seventh larvae from each family on each diet that molted into the 4th instar were reared through to pupation on their respective diets (*N*=448 larvae; hereafter "egg-to-pupa larvae"). The remaining 3 larvae were discarded. After molting into the 4th instar, the 4 egg-to-pupa larvae were placed together in an 11-cm Petri dish and provided fresh food every 3 d or more often if necessary until pupation.

Early instar survivorship, defined as survivorship from hatching through the 3rd instar, was measured for all larvae, while late instar survivorship, defined as survivorship from the 4th instar through pupation, was measured only for the 448 egg-to-pupa larvae. Since larvae reared in the same cup are not independent replicates, early instar survivorship was first calculated for the 10 larvae from the same family on a given diet. Since survivorship was always at least 80%, differences among diets in early survivorship were determined by comparing the number of families with 100% vs. 80–90% survivorship by a *Chi-square* test. Similarly, differences among diets in late instar survivorship were determined by comparing the number of families with 100% vs. 75% survivorship by a *Chi-square* test. For both early and late instar survivorship, a Yates correction was applied when one or more expected value was less than 5.

Effect of Phytoestrogens on Growth The effect of genistein and daidzein on gypsy moth growth was determined in two ways. Egg-to-pupa growth rate (EPGR) was calculated for each of the 442 egg-to-pupa larvae that survived to pupation by drying pupae to constant weight at 60°C and

dividing the final pupal dry weight by the number of days from hatching to pupation. The initial dry weight of all larvae was assumed to be negligible. Upon pupation, the fresh weight of each pupa was also measured, and compared to the pupal dry weight to calculate pupal percent water. Since larvae reared in the same dish are not independent replicates, all measures were averaged separately for males and females sharing a dish (i.e., larvae of the same sex and from the same family on a given diet), and averages were compared among diets by ANOVA or, if data were not normal or variances were unequal, by Kruskal-Wallis tests. Sample sizes are slightly less than 28 because, on each diet, at least one dish yielded only male or female pupae.

Relative growth rate during the 4th instar and its components (consumption rate, digestive efficiency, and conversion efficiency) were measured for one larva per family per diet. As described above, the fifth larva from each family on each diet to molt to the 4th instar was used to in this experiment (N=28 larvae per diet). We chose the 4th instar since this is the last instar that does not end with pupation for either sex; males pupate after five instars and females pupate after six instars.

Upon molting into the 4th instar, each test larva was weighed and placed into a new 25-ml polystyrene cup containing a weighed amount of control, genistein, daidzein, or genistein+daidzein diet. Fresh diet was provided every other day. Upon molting into the 5th instar, each larva was weighed, frozen, dried to constant weight at 60°C, and reweighed. All uneaten food and all frass produced during the instar were also dried and weighed. Larval performance measures (Waldbauer, 1968; Karowe and Golston, 2007), based on dry weights, were calculated as follows:

Relative consumption rate(RCR)

$$= \text{food ingested}/(\text{average larval weight})(\text{days})$$

Approximate digestibility(AD)

$$= (\text{food ingested} - \text{frass})/\text{food ingested}$$

Efficiency of conversion of digested food(ECD)

$$= \text{weight gained}/(\text{food ingested} - \text{frass})$$

Efficiency of conversion of ingested food(ECI)

$$= \text{AD} \times \text{ECD}$$

Relative growth rate (RGR)

$$= \text{weight gained}/(\text{average larval weight})(\text{days})$$

The effect of diet on nutritional indices and other measures of 4th instar performance (instar duration, final dry weight, and weight gained and frass produced during the 4th instar) was determined by one-way ANOVA followed by Tukey pairwise comparisons if data were normal and variances were equal, or by Kruskal-Wallis tests followed by pairwise Mann–Whitney U test if the assumptions of ANOVA were not met.

Results

Effect of Phytoestrogens on Survivorship Early instar survivorship ranged from 97.5% to 100% and did not differ significantly among the four diets (χ^2=6.89, $d.f.$=3, P=0.075; Fig. 2a). Late instar survivorship ranged from 99.1% to 100%. Since only a single late instar larva died, no statistical analysis was possible; however, it is clear that late instar survivorship did not differ among diets (Fig. 2b). It is unlikely that inclusion of the three discarded larvae per family per diet would have resulted in differences among diets. Although these were the slowest growing larvae, the great majority were growing nearly as well as their siblings. Across diets, only 0% to 3.7% of discarded larvae were in the 2nd instar, while 96.3% to 100% were in the 3rd instar (χ^2=3.4, $d.f.$=3, P>0.3).

Effect of Phytoestrogens on Egg-to-Pupa Growth Among females, measures of egg-to-pupa performance did not differ significantly among diets (Table 1). Egg-to-pupa growth rate ranged from 7.45 to 7.79 mg/day (P=0.49), the number of days from hatching to pupation ranged only from 53.9 to 54.5 (P=0.35), pupal dry weight ranged from 40.3 to 42.3 mg (P=0.45), and pupal water content ranged only from 77.2 to 77.6% (P=0.38).

Similarly, among males, most measures of egg-to-pupa performance did not differ among diets (Table 1). Egg-to-pupa growth rate ranged from 2.34 to 2.49 mg/day (P=0.41), the number of days from hatching to pupation ranged only from 48.0 to 48.7 (P=0.76), and pupal dry weight ranged from 11.3 to 12.1 mg (P=0.39). However, the water content of male pupae differed among diets (χ^2=8.16, $d.f.$=3, P=0.043). Water content was significantly higher on the genistein+ daidzein diet than on the control, genistein, and daidzein diets.

Effect of Phytoestrogens on 4th Instar Performance Measures of 4th instar performance did not differ among larvae reared on control vs. phytoestrogen-containing diets (Table 2). No effect of diet was observed for any of the nutritional parameters, which generally differed by less than 5% among diets. Larger, but still nonsignificant, differences were noted for final dry weight, which differed by 17%
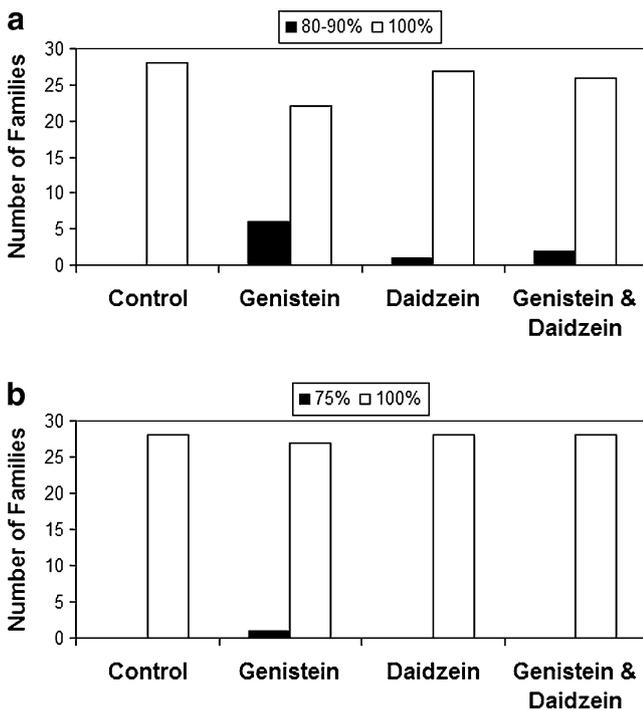
Fig. 2 (**a**, *top*) Early survivorship of gypsy moth (through the 3rd instar), expressed as the number of families on each diet that had 80–90% vs. 100% survivorship and (**b**, *bottom*) late survivorship (4th instar through pupation), expressed as the number of families on each diet that had 75% vs. 100% survivorship

among diets, total weight gained during the feeding trial, which differed by 20% among diets, and total food eaten during the feeding trial, which differed by 24% among diets.

Discussion

The results of this study indicate that the two most widely studied phytoestrogens, genistein and daidzein, have no dietary effect on a generalist herbivore, the gypsy moth. Even when these compounds were incorporated into an artificial diet at levels far above those normally found in leaves, gypsy moth survivorship was nearly 100%. Moreover, none of the measures of performance during the entire larval stage or during the 4th instar were compromised by genistein or daidzein, alone or in combination. Therefore, the results of this study do not support the hypothesis that these phytoestrogens occur in aboveground plant tissues and defend against generalist insect herbivores.

Previous studies have detected antifeedant and/or antibiotic effects of genistein and/or daidzein. For instance, when applied to agar-cellulose disks, genistein reduced consumption by the root-feeding scarab beetle, *Heteronychus arator* (Sutherland et al., 1980). Similarly, when applied to leaf

disks, daidzein adversely affected growth and survivorship of the cabbage looper, *Trichoplusia ni* (Sharma and Norris, 1991). Unfortunately, since the weight of disks was not reported, it is not possible to determine whether these herbivores ingested phytoestrogens at levels known to occur in nature. We are aware of only one study in which it is possible to calculate exposure level. Wang et al. (1998) observed deterrence of redlegged earth mites, *Halotydeus destructor*, by a genistein-containing solution, but only at concentrations of 0.045% and above. Assuming the test solution had a density of approximately 1 g/ml, this concentration is more than five times the maximum genistein level reported from soybeans (Mazur and Adlercreutz, 1998; Dixon, 2004). It is not clear that adverse effects previously reported in the literature provide strong evidence of anti-herbivore effects of genistein or daidzein.

Consistent with our results, several studies have suggested that genistein does not possess anti-herbivore activity. For instance, genistein did not reduce feeding by scarab beetles *Costelytra zealandica* (Sutherland et al., 1980; Lane et al., 1987) or *Heteronychus arator* (Lane et al., 1987). In a related study (Lane et al., 1985), genistein did not reduce feeding by *C. zealandica* when incorporated into agar-cellulose disks at 0.2 mg/g, a concentration known to occur in soybean (Dixon, 2004). The authors suggested that this lack of activity might be due to the absence of an oxy function at the 2′ position of the B ring and, even more importantly, the absence of a B ring cyclic isoprenoid moiety.

It should be noted that most flavonoids and isoflavonoids exist in plant tissues primarily as glycosides rather than as aglycones such as genistein and daidzein. However, it is likely that in insects, as in humans, glycosides are readily converted into aglycones by β-glucosidases in the gut (Matsuura and Obata, 1993; Steensma et al., 1999) although that conversion has not been demonstrated for insects.

If genistein and daidzein do not provide effective defense against insect herbivores, then why are they present in aboveground plant parts? Phytoestrogens may defend plants by reducing fertility in herbivorous mammals (Jefferson et al., 2009) or seed-eating birds (Rochester and Millam, 2009), or by conferring resistance to fugal (Naoumkina et al., 2007) or viral (Andres et al., 2009) pathogens.

It is premature to rule out the possibility that phytoestrogens are in some cases effective defenses against insect herbivores. There was a slight indication in this study that genistein may decrease early instar survivorship ($P=0.075$; Fig. 2a), although the increase in mortality was very small. Furthermore, since it is highly polyphagous, the gypsy moth may be less susceptible than most insect herbivores to adverse effects of phytoestrogens. Although there is no reason to believe that gypsy moth encounters genistein or daidzein in its natural diet, it

Table 1 Egg-to-pupa performance (Mean±S.D.) for female and male gypsy moth larvae reared on control or phytoestrogen-containing diets

Females[a]					
Diet	N	Egg-to-Pupa Growth Rate (mg/d)	Development Time (days)	Pupal Dry Weight (mg)	Water Content (%)
Control	25	7.45±0.76[a]	54.2±1.6[a]	40.3±4.2[a]	77.6±0.7[a]
Genistein	27	7.77±0.94[a]	54.5±1.4[a]	42.3.±4.5[a]	77.2±0.8[a]
Daidzein	23	7.63±0.84[a]	53.9±1.4[a]	41.1±4.7[a]	77.5±1.0[a]
Genistein+Daidzein	26	7.79±0.96[a]	53.9±1.2[a]	41.9±4.8[a]	77.5±0.9[a]
$F_{3,97}$		0.82	1.11	0.88	1.05
P-value		0.49	0.35	0.45	0.38
Males					
Diet	N	EPGR (mg/d)	Development Time (days)	Pupal Dry Weight (mg)	Water Content (%)[a]
Control	25	2.34±0.30[a]	48.0±3.1[a]	11.3±1.8[a]	79.3±2.0[a]
Genistein	25	2.49±0.36[a]	48.7±2.7[a]	12.1±1.9[a]	79.0±1.4[a]
Daidzein	27	2.47±0.35[a]	48.2±1.9[a]	11.9±1.9[a]	79.1±1.9[a]
Genistein+Daidzein	26	2.46±0.34[a]	48.5±3.0[a]	11.6±2.3[a]	78.4±2.3[b]
Test statistic[b]		0.96	0.39	1.01	8.16
P-value		0.41	0.76	0.39	0.043

[a] Means followed by the same letter are not significantly different at $P<0.05$

[b] Significance of differences determined by a Kruskal-Wallis test followed by pairwise Mann–Whitney U tests since data could not be normalized

[c] F statistic for ANOVA or χ^2 statistic for Kruskal-Wallis test

Table 2 Nutritional indices (Mean±S.D.) for 4th instar gypsy moth larvae fed control or phytoestrogens-containing diets

Diet	N	RCR (g/g/d)[a]	AD (%)[a]	ECD (%)[a]	ECI (%)[a]	RGR (g/g/d)
Control	22	0.438±0.086	51.6±5.6	60.4±11.4	30.6±3.6	0.132±0.013
Genistein	25	0.416±0.039	51.6±4.1	61.5±6.7	31.5±1.5	0.131±0.011
Daidzein	23	0.419±0.046	50.8±2.7	62.1±6.0	31.8±2.8	0.133±0.014
Genistein+Daidzein	23	0.437±0.058	50.6±5.2	60.4±4.3	30.4±2.8	0.132±0.009
Test statistic[b]		1.72	0.95	1.28	2.41	0.13
P-value		0.63	0.81	0.73	0.49	0.94

Diet	N	4th Instar Duration (days)	Final Dry Weight (g)[a]	Weight Gained (g)[a]	Food Eaten (g)[a]
Control	22	9.13±0.84	0.059±0.019	0.045±0.017	0.149±0.062
Genistein	25	9.02±0.76	0.057±0.017	0.042±0.014	0.135±0.046
Daidzein	23	9.13±0.84	0.054±0.015	0.041±0.013	0.131±0.041
Genistein+Daidzein	23	9.35±0.78	0.063±0.017	0.049±0.014	0.162±0.052
Test statistic		0.69	3.57	2.45	4.58
P-value		0.56	0.31	0.48	0.21

[a] Significance of differences determined by a Kruskal-Wallis test because data could not be normalized

[b] F statistic for ANOVA or χ^2 statistic for Kruskal-Wallis test

RCR Relative consumption rate

AD Approximate digestibility

ECD Efficiency of conversion of digested food

ECI Efficiency of conversion of ingested food

RGR Relative growth rate

does encounter the structurally similar compounds rutin and quercetin (Liebhold et al., 1995; Perić-Mataruga et al., 1997). However, since both rutin and quercetin inhibit development of gypsy moth caterpillars (Beninger and Abou-Zaid, 1997; Peric-Mataruga et al., 2001), it is unlikely that gypsy moths possess a broad ability to detoxify isoflavones. Our results, however, indicate that genistein and daidzein do not affect this insect herbivore. Therefore, it seems unlikely that the primary function of these compounds in aboveground plant tissues is deterrence of generalist herbivores.

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