**Leaf herbivory induces resistance against florivores in
*Raphanus sativus***

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Abstract

Florivory can have significant negative effects on plant fitness, driving selection for resistance traits in flowers. In particular, herbivory to leaves may induce resistance in flowers because herbivores on leaves often become florivores on flowers as plant ontogeny proceeds. The literature on inducible resistance in floral tissues is limited, so we used a series of experiments to determine if prior leaf damage by *Spodoptera exigua* caterpillars affected florivore preference and performance on wild radish (*Raphanus sativus* L.). We found that *S. exigua* larvae preferred petals from control plants versus petals from plants exposed to prior leaf damage, and that larvae gained more weight on petals from control plants, although this depended on the presence of anthocyanins in the petals. Our results suggest that leaf damage can induce changes in petals that reduce *S. exigua* larval fitness.

Key words: anthocyanins, floral herbivory, florivory, induced resistance, *Raphanus sativus*

39 **Introduction**

40 Many different types of herbivores may feed on a plant during its growth and
41 development. Some of these herbivores specialize on particular parts of the plant,
42 while others are more general in their preferences. For example, some herbivores
43 primarily feed on roots, some on shoots, and others on floral tissues. Florivores,
44 those animals feeding on floral tissue, are some of least studied among herbivores
45 (McCall and Irwin 2006). These organisms consume floral tissues before
46 maturation of the seed, and thus can have direct and indirect effects on overall plant
47 fitness.

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49 Florivores may eat or damage the primary reproductive organs in a flower
50 (Krupnick and Weis 1999). This can result in nearly complete seed loss in some
51 plant populations (Washitani et al. 1996). Other types of damage may affect tissues
52 involved in pollinator signaling, like petals (McCall 2008). Damage to these tissues
53 can result in reduced reproduction through reduced pollinator service and/or seed
54 set (Karban and Strauss 1993; Lohman et al. 1996; Cardel and Koptur 2010; see
55 González-Browne et al. 2016 for a meta-analysis). Given the potential fitness costs
56 of florivory, plants may have evolved to limit the amount of damage caused by
57 florivores through physical and/or chemical resistance. One framework that can
58 explain the distribution of these traits is the Optimal Defense Theory (ODT) (McKey
59 1974; Rhoades 1979). ODT assumes that there is a substantial cost to producing
60 resistance traits, such that all tissues cannot be maximally protected at all times.
61 Given this tradeoff, ODT predicts that resistance should be higher in tissues that are

more valuable, more likely to be attacked by herbivores, or are less costly to defend (McKey 1974; Rhoades 1979).

Plant resistance may also vary depending on herbivore activity. Constitutive resistance is active without an initial trigger, whereas induced resistance is only activated after an initial bout of herbivore damage or some other trigger (Karban and Baldwin 1997). Induced resistance is thought to be less costly than constitutive resistance because it is not activated if initial damage does not occur. Thus, ODT predicts that less valuable tissues will be protected with inducible resistance, whereas more valuable tissues would be protected with constitutive resistance, all other factors being equal (Karban and Nagasaka 2004). Induced resistance in flowers is not without costs, though, as induction by leaf damage can reduce pollinator attractiveness in *Solanum peruvianum* (Kessler et al. 2011). In another example, natural leaf damage to *Sinapis arvensis* affected both pollinator preference and the pollinator-plant community networks that included *S. arvensis*, possibly due to changes in floral volatiles (Hoffmeister et al. 2016).

Flowers might be expected to have high levels of constitutive resistance because they are directly involved in fitness and are usually highly apparent to visual herbivores (Zangerl and Rutledge 1996; Strauss et al. 2004; McCall and Irwin 2006). Researchers have found inducible responses in flowers. In particular, damage to leaves can lead to increased resistance in flowers. This mechanism may be adaptive if the same leaf herbivores also eat flowers, which has been reported in the

literature (e.g. Agerbirk et al. 2010; Merwin and Parrella 2014; Abdalsamee and Müller 2015). In some systems, a leaf herbivore first eats leaves and then moves up a stem to feed on flowers or inflorescence tissue (Smallegange et al. 2007; Bandeili and Müller 2010). Under these circumstances, a plant may be selected to defend its reproductive tissues after initial damage to its leaves because leaf damage would be a reliable predictor of floral damage. For example, leaf damage can increase floral nicotine levels in *Nicotiana attenuata* (Euler and Baldwin 1996). McCall and Karban (2006) showed that artificial leaf damage and methyl jasmonate were able to deter florivory in *N. attenuata*. In *Raphanus sativus*, leaf damage increased total glucosinolate concentrations in flowers (Strauss et al. 2004). Researchers have also found inducible chemicals in floral nectar. Halpern et al. (2010) showed that leaf damage can increase nicotine concentrations in *Nicotiana quadrivalvis* nectar and Adler et al. (2006) showed that leaf damage increased alkaloid levels in *Nicotiana tabacum* nectar.

Despite these gains, past studies focused solely on tissue chemistry, and no research to date has addressed whether induced resistance in floral tissue has an actual effect on florivore preference and performance. For selection to favor induced resistance in flowers, there should be a negative effect of induction on florivores, especially if the florivores are also leaf herbivores early in plant ontogeny. As of yet, there is no work investigating if induced resistance, triggered by leaf damage, can negatively affect florivore performance.

Genetic variation within populations is also necessary for natural selection of induced resistance in flowers. Researchers have found significant genetic variation in induced resistance in leaves, although few have investigated how genetic variation affects induced resistance in floral tissues. In a rare example, Strauss et al. (2004) found that although different families differed in glucosinolate concentrations in *Raphanus sativus*, the strength of induction across petals and leaves did not depend on maternal family. Because of the paucity of studies on the variation in floral induction strength within populations, we investigated whether induced resistance differs within maternal families in a single population.

The above studies considered genetic variation within single populations, but the strength of induction observed in one particular population may not be generalizable to other populations. In fact, there is reason to expect induction to vary across plant populations, especially if the type of herbivores and the severity of their damage varies across those populations (Karban and Nagasaka 2004). To our knowledge, no one has investigated whether different plant populations differ in their strength of induced resistance in floral tissue or if induced resistance, in general, affects florivore performance.

Using the model species *Raphanus sativus*, we consider several questions related to the aforementioned gaps in our knowledge about induced resistance in flowers. First, we ask whether prior damage to leaves affects florivore preference of petals. Second, we ask if prior leaf damage affects florivore performance, as measured by

131 weight, on petals among maternal families within a single population. Third, we ask
132 if the effects of leaf damage on florivore performance differ among several plant
133 populations and if petal anthocyanin presence affects induced resistance.

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Methods

Plant system

Raphanus sativus L. is an annual plant produced from crosses between *R. raphanistrum* and agronomic *R. sativus* cultivars (Hegde et al. 2006). Its seeds germinate during the first part of the wet season in California's Mediterranean climate (October–December), with plants flowering between March and July. Floral color phenotype (bronze, pink, white, and yellow) is controlled by two independently segregating loci (Panestsos 1964; Irwin et al. 2003). Leaf herbivores include *Plutella xylostella* (Plutellidae) and *Platyrepia virginalis* (Erebidae), both of which also can feed on flowers. On a single plant, *Pl. virginalis* and *P. xylostella* individuals can be both leaf herbivores and florivores (A. McCall, personal observation).

Florivore choice and performance procedures

We used the same general procedure to test florivore preference and performance for the three experiments described below. We attempted to induce resistance responses in *R. sativus* flowers by damaging the leaves of experimental plants with *Spodoptera exigua* larvae, a generalist herbivore often used for bioassays. Half of the plants were randomly chosen to receive herbivore damage by caging a neonate *S. exigua* larva on the third true leaf. Control plants received a cage without any larvae. We allowed the larvae on the experimental plants to feed until half of the leaf area was consumed. Thus, every damaged plant received the same relative amount of damage. We then removed all cages from control and experimental plants and allowed the plants to grow until they flowered about 2 weeks later. Very little

work on the duration of induction in *Raphanus* has been conducted, but Agrawal (1999) showed that induction of resistance in leaves may last 6 weeks in *R. raphanistrum*.

For the preference test, a single petal from a randomly-chosen control and experimental plant of the same color morph was selected and placed in a 120 mm Petri dish, along with a moist ball of tissue paper to ensure that the petals would remain fresh for consumption. For the performance tests, we selected two petals from the newest flower on a plant and placed them in the arena with a moist tissue paper ball and a neonate larva. Petals and tissue paper balls were replaced every two days and petals always came from the same plant for each arena. The only exception to this procedure was if the larvae ate all of the available petal tissue before 2 days. If this happened, we added 2 more petals when the food supply was exhausted. During the replacement of tissue, we weighed the petals to ensure that constant weights were maintained among and within individual arenas. If weights were not within 0.005g of each other on successive days of the tests, we selected new petals until the desired mass was achieved. We allowed the larvae to feed for 24h for the preference test and for 10 days for the performance tests.

Preference

Forty *R. sativus* plants from five dams in the Jepson Prairie population (Table 1) were grown in a greenhouse in September of 2008. After growing the experimental and control plants as described above, we set up feeding trials where each larva

could feed on petals obtained from damaged and undamaged plants. We allowed the larvae to feed for 24h, after which we scored which flower disk had the most damage. One larva escaped and we were unable to determine which disk had the most damage in one arena, leaving us with 18 total replicates. We tested preference using a Chi-square goodness-of-fit test.

Performance: single population

We also wanted to know if induction of resistance in petals or anther tissue could affect *S. exigua* performance, measured as total weight gain. We employed a bioassay method using *S. exigua* and plants from a population at Jepson Prairie, California (Lat.: 38.303°; Long.: 121.824°). On May 15, 2009, seeds from this population were collected by placing a 20m linear transect along the side of the road where the bulk of the population was located. Silique-bearing plants at every 2m were selected for collection, although there were only eight total plants that set fruit in that year. Approximately 30 siliques from each maternal plant were haphazardly collected and placed in paper bags at room temperature until germination for the experiment. On November 10, 2009, five seeds from five different siliques per maternal plant were planted in potting soil. Plants were grown under a 16h day: 8h night regime in a pest-free greenhouse until the third true leaf was fully expanded on each plant, when we applied the initial damage treatment. As the plants flowered we also noted whether they expressed anthocyanins in their petals (bronze and purple) or if they did not (white and yellow).

We tested the performance of neonate *S. exigua* larvae using the methods described above, feeding larvae petals from control or treatment plants. Initially we planted 70 plants, with 36 plants randomly assigned to the control treatment and 34 plants randomly assigned to the induction treatment. During the course of the feeding trials 10 larvae died, resulting in 60 total replicates. There was no association of larval death with a particular treatment (Chi-square = 1.611, $P = 0.20$). Maternal family 1 had 14 replicates, family 2 had 16 replicates, family 3 had 11 replicates, and family 4 had 19 replicates. Fifty larvae were reared on pink-flowering plants and ten were reared on white-flowering plants.

The data exhibited homogeneity of variances and were normally distributed, so we performed a mixed-model ANOVA with the dependent variable of larval weight and the independent variables of planting block, maternal family, damage treatment, and the interaction between maternal family and damage treatment. We considered planting block as a random effect and maternal family a fixed effect because we sampled a large proportion (approximately 50%) of this population.

Performance: multiple populations

In order to test whether the effects of prior leaf damage are dependent on the population being tested and to see if the presence of anthocyanins affected the results, we conducted an experiment where we manipulated leaf damage on plants from several populations of *R. sativus*. These populations were a subset of those used in an earlier experiment that investigated whether variance in leaf damage was

related to inducibility of defense in leaves (Karban and Nagasaka 2004). Those populations in Karban and Nagasaka (2004) were originally chosen because they spanned a climatic gradient from wet areas to dry areas in Northern California.

We planted 10 seeds from each of 12 *R. sativus* populations in a pollinator-free greenhouse. Nine seeds failed to germinate, leaving us with 111 plants for the experiment. We again produced experimental and control plants using the general procedure outlined above. During the course of the experiment 34 larvae died, so we were left with 77 living larvae at the end of 10 days. There was no difference in the proportion of larvae that died between the damage treatments ($X^2 = 1.54$, 1df, $P = 0.22$). We then reduced the data set further by excluding those populations (CHER, DIXO, NAPA, PLIN, STRW, see Table 1) that did not have at least two replicates of each treatment or anthocyanin morph. This left us with 58 total plants in seven populations for the final analysis (Table 1). There were 34 control plants and 24 experimental plants and 28 with anthocyanins present in their flowers versus 30 plants with anthocyanins absent from their flowers.

The data exhibited homogeneity of variances and residuals were normally-distributed, so we performed a mixed-model ANOVA to test if planting block, population, anthocyanin presence in petals, population by treatment or population by anthocyanin had significant effects on larval weight gain after 10 days of feeding. We considered the population, population by treatment, and planting block as random effects. We determined the significance of random factors by examining the

253 95% CI; if these intervals included zero, then the random effect was not considered
254 significant at the $\alpha = 0.05$ level. We included anthocyanin presence in the petals
255 (A+/A-) and the interaction between anthocyanin presence and the treatment effect
256 because different color morphs are known to induce different levels of
257 glucosinolates (Strauss et al. 2004).

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259 All analyses were performed using JMP Pro v.12 (SAS Institute, Cary, NC USA)

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Results

Preference

S. exigua larvae preferred petals from undamaged plants in 15 out of 18 (83.3%) trials. This number was significantly different from the numbers expected from the null hypothesis of no preference between the treatments ($\chi^2 = 8.0$, 1df, $P = 0.005$).

Performance: single population

For insects reared on petals, there was a significant effect of planting block (variance component = $1.01\text{e-}8$, $P < 0.05$) and no significant effect of the damage treatment ($F_{1,51} = 2.96$, $P = 0.09$), or maternal family ($F_{3,51} = 0.38$, $P = 0.77$) on larval weight after ten days. There was a strong trend for an interaction between damage treatment and maternal family ($F_{3,51} = 2.76$, $P = 0.051$). On examining the simple effects of damage treatment within each maternal family, there were no significant effects of damage in families 1 ($F_{1,51} = 2.42$, $P = 0.13$), 2 ($F_{1,51} = 0.77$, $P = 0.38$), or 4 ($F_{1,51} = 2.38$, $P = 0.12$), but larvae from control treatments weighed 79% more than larvae from damage treatments in family 3 ($F_{1,51} = 4.69$, $P = 0.03$, see Figure 1).

Performance: multiple populations

Planting block in the greenhouse had a significant effect on larval weight (variance component = $1.51\text{e-}8$, $P < 0.05$). There was no significant effect of population (variance component = $8.19\text{e-}9$, $P > 0.05$), and no significant population by treatment effect (variance component = $-1.00\text{e-}8$, $P > 0.05$). For fixed effects, there was a significant effect of the leaf damage treatment, such that larvae on damaged plants

285 weighed 18% less than larvae on undamaged plants ($F_{1,9.01} = 6.42, P = 0.03$) and no
286 significant effect of petal anthocyanins on larval weight ($F_{1,37.18} = 0.84, P = 0.36$).
287 There was a significant leaf damage by petal anthocyanin interaction ($F_{1,13.3} = 5.59, P$
288 $= 0.03$). Examining the simple effects within anthocyanin-free morphs, we found
289 that larval weights were significant lower on petals from damaged plants than on
290 petals from undamaged plants ($F_{1,19.5} = 9.66, P = 0.006$). In contrast, there were no
291 significant effects of leaf damage on larval weight in morphs containing
292 anthocyanins in their petals ($F_{1,5.43} = 0.012, P = 0.92$, see Figure 2).

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Discussion

If induced resistance occurs in petals, we might expect that florivores to choose petals from undamaged versus damaged plants. We found evidence that leaf damage can alter the preference for petals. Specifically, florivores preferred petals from control plants versus petals from plants with prior leaf damage. This finding is consistent with the idea that insects can initially reject petals due to induced secondary compounds in the flowers, physical changes in petal structure, or changes in the nutritional composition of the flowers. To our knowledge, this is the first evidence showing that florivores show a preference for petals from undamaged versus damaged plants. Likewise, larvae reared on petals from damaged plants gained significantly less weight than those larvae reared on petals from control plants. We observed this both within a single population and across multiple populations, although this depended on the anthocyanin content in the latter case. These results suggest that either signals from damaged leaves or defensive chemicals themselves are able to travel to flowers after initial herbivore damage and can have negative effects on florivore health.

Other researchers have found that prior damage to plant tissue can also affect floral visitors, although few have documented a negative fitness effect on florivores. For example, McCall and Karban (2006) found that both insect damage and application of methyl jasmonate on leaves decreased florivore damage in *Nicotiana attenuata*. In *Nemophila menziesii*, McCall (2006) showed that initial damage to flowers could reduce florivory in later flowers, but they did not examine florivore performance.

Our current work suggests that plants can employ less costly inducible resistance in petals. Our results could be explained by ODT if flowers, and petals in particular, are relatively cheap to produce or if more constitutive resistance may negatively affect pollination (McCall and Irwin 2006). *Raphanus sativus* plants often make hundreds of flowers (A. M., personal observation) so that the sheer number of flowers available for reproduction may mitigate the cost of losing petal tissue when florivore pressure is relatively low. This, in turn, might preclude the selection for costly constitutive chemical defenses in the flowers. Another reason that *R. sativus* might be selected to induce resistance in flowers is because induction is adaptive with respect to florivore behavior. For example, if florivores start their lives on basal leaves, they can move acropetally to the top of the plant, where inflorescences will be at risk for damage. This type of movement has been observed in larvae feeding on other Brassicaceae (Smallegange et al. 2007; Bandeili and Müller 2010), so it is possible that *R. sativus* may induced resistance against similar florivores.

We also found evidence that induction of resistance varies with maternal family in the Jepson population. This may not be surprising, as Agrawal et al. (2002) found significant additive genetic variation in the strength of induction in *Raphanus raphanistrum* leaves. It would be interesting to investigate whether those genotypes that display high levels of induced resistance in leaves also have high levels of induction in flowers.

343 Interestingly, we found no evidence that induced resistance in flowers varied
344 among populations of *R. sativus*, even though the type and intensity of herbivory did
345 vary across the populations (see Table 1). This is in contrast to Karban and
346 Nagasaka's (2004) findings that populations of *R. sativus* along a climatic gradient in
347 Northern California exhibited significant variation in induction of resistance in
348 leaves. Our results may differ from this study for at least two reasons. First, our
349 sample sizes per population were much lower, with an average of 8 plants per
350 population, so it is possible that we did not have enough statistical power to detect
351 differences in induction. Second, it is possible that the variation in induced
352 responses in flowers does not necessarily reflect the amount of variation in
353 induction for leaves across these populations. This may be true in *R. sativus*, where
354 researchers found that floral glucosinolates were not as inducible as leaf
355 glucosinolates within a population (Strauss et al. 2004).

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357 We also found that induction of resistance in petals depended on the presence of
358 anthocyanins in petals in the multiple population experiment. In particular, induced
359 resistance was only found in anthocyanin-free petals. This could be due to
360 pleiotropic action of genes involved in producing glucosinolates and the precursors
361 to anthocyanins (Hemm et al 2003). Strauss et al. (2004) found that white and pink
362 flowers had similar constitutive concentrations of glucosinolates, but that
363 glucosinolates were induced at higher levels in pink *R. sativus* flowers than in the
364 white morphs. This is in contrast to our findings, and could be explained because
365 we used different populations of *R. sativus* than Strauss et al. (2004) or that our

methods of induction were not identical. Alternatively, glucosinolate induction in pink morphs as shown by Strauss et al. (2004) may not necessarily translate into reduced performance of florivores relative to the other color morphs.

Although we showed that leaf damage has negative effects on florivores, we cannot necessarily conclude that induction is an adaptation to reduced flower damage in *R. sativus*. Alternatively, damage to leaves may increase levels of defensive chemicals, reduce nutrient concentration or availability, or affect some other physical attribute in the entire plant, with effects on florivores being a nonadaptive side effect of the leaf damage. This hypothesis would mirror Adler's (2000) nonadaptive hypothesis regarding correlated concentrations of resistance compounds in leaves and nectar. Manson et al. (2012) found that pooled concentrations of cardenolides across 12 *Asclepias* species were positively correlated in nectar and leaves, possibly giving support to the nonadaptive hypothesis. Alternatively, the authors also found that the qualitative profiles of cardenolides were significantly different among nectar and leaf samples.

Conclusions

We show that *S. exigua* larvae both prefer petals from control plants and perform better on control plant petals than on damaged plant petals. This effect is dependent on maternal family identity in at least one population, and across multiple populations the effect was only seen in plants without anthocyanins in their petals. These results suggest that plants may protect themselves from leaf and floral

389 damage through induction of resistance stimulated by relatively small levels of
390 initial leaf damage. Whether or not this effect can increase fitness of *R. sativus* under
391 field conditions remains an open, and important, question.

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Table 1. Locations of populations of *Raphanus sativus* where seed was originally collected. Bolded populations indicate those populations that were used in the multiple populations experiment. The remaining populations did not have enough variation in petal anthocyanins to include in the final analysis.

Population	Latitude	Longitude
CHER	38.336	121.022
CONS	38.2575	121.441
DIXO	38.361	121.824
HOOD	38.364	121.510
NAPA	38.221	122.238
PEDR	38.511	121.804
PLIN	38.584	121.732
SNOD	38.2772	121.5008
SONO	38.2356	122.5292
STON	38.4083	121.4892
STRW	38.563	121.800
YOLO	38.557	121.672

Figure Legends

Fig. 1 The effects of maternal family and leaf damage treatment on the weights of *Spodoptera exigua* larvae after 10 days of feeding on petals. Black bars represent larval weights from control plants and grey bars represent larval weights from the leaf damage treatment. Bars represent means $\pm$ 1 SE.

Fig. 2 The effect of leaf damage and petal anthocyanin content on *Spodoptera exigua* weight after 10 days of feeding on petals. Plants were grown from seven different populations in Northern California (see Table 1). Black bars represent larval weights from control plants and grey bars represent larval weights from the leaf damage treatment. Bars represent means $\pm$ 1 SE.

537 **Fig. 1**

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539

540 **Fig. 2**

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