

To eat or not to eat:

Larval feeding behavior and leaf components that affect the survival of *Pieris macdunnoughii* on the invasive mustard *Thlaspi arvense*

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Abstract

Pieris macdunnoughii, a butterfly native to the Rock Mountains in Colorado, is a specialist on mustard plants. An invasive mustard, *Thlaspi arvense*, introduced approximately 100 years ago creates an evolutionary trap for *P. macdunnoughii* due to the chemical signals that encourage ovipositioning, but it does not support larval development. Such interactions suggest larvae will develop means to successfully feed on *T. arvense*. First instar feeding behavior on *T. arvense* was compared to behavior on native host plants, *Cardamine cordifolia* and *Descurainia incana*. In laboratory trials, larvae were given *T. arvense* or *C. cordifolia* and photographed in two hour intervals for gut content. The results show that larvae fill their guts soon after encountering *C. cordifolia*, but for *T. arvense* larvae eat only small amounts of leaves and hardly fill their guts, exhibiting deterrent properties. Similar patterns of herbivory were observed in field trials where larvae were placed on either *T. arvense* or *C. cordifolia*, though larvae in the lab filled their guts more than those in the field. In a feeding assay with sinigrin, a glucosinolate believed to be the precursor in a metabolic pathway that produces hydrogen cyanide, 50 μ mol of sinigrin per gram of leaf mass were added to *T. arvense*, *C. cordifolia*, and *D. incana* leaves and fed to larvae along with control leaves painted with water. The results suggest larvae perform best on *D. incana* despite added sinigrin and sinigrin has deterrent effects for all species and increases larval death.

Introduction

The insect order Lepidoptera exhibits a variety of life history traits, these include methods for laying eggs and locating larval host plants. In some species, first instar mobility and sensory features such as eyesight are severely limited and their survival depends on the ability of female butterflies to correctly oviposit eggs on suitable host plants. If incorrectly placed, the young larvae are incapable of transferring to a different plant and die as a result (Scott 1986). This is true for *Pieris macdunnoughii* larvae, a member of the Pieridae family. Within the Pieridae, many butterflies have specialized to recognize mustard (Brassicaceae) host plants. For *P. macdunnoughii*, a native species to the southern Rocky Mountains that flies in moist meadows, studies have observed their ovipositioning behavior and found that females only lay eggs on mustard plants (Chew 1977). These include *Descurainia incana*, *Arabis drummondii*, *Cardamine cordifolia*, *Thlaspi montanum*, and *Thlaspi arvense*; meanwhile, the females avoid and do not lay eggs on *Erysimum asperum* (Chew 1977). Feeding trials show that these mustards are not equal host plants. Larvae fed various mustards were found not to survive on *E. asperum* or *T. arvense* with no larvae making it to pupation (Chew 1975). Larvae made it to pupation on the species: *D. incana*, *T. montanum*, *A. drummondii*, *D. aurea*, and *C. cordifolia* (Chew 1975). Among these species, *C. cordifolia* produced the largest larvae by mass (Chew 1975).

As the taxa Brassicaceae is distinguished by glucosinolates, a chemical profile of these mustard species revealed a correlation between the larval behavior on the different host plants and the glucosinolates present (Rodman and Chew 1980). The similar feeding and oviposition behaviors in *A. drummondii*, *C. cordifolia*, and *T. montanum* were matched with the presence of isopropylglucosinolate, 2-butylglucosinolate and their hydroxy forms. *E. asperum* was distinguished by its predominate glucosinolate, 3-methylsulfinylpropyl (Rodman and Chew

1980). The third group was characterized by the presence of allylglucosinolate, commonly known as sinigrin, found in *D. incana* and *T. arvense* (Rodman and Chew 1980). Sinigrin has been found to stimulate egg laying in *P. rapae* and *P. macdunnoughii* (Renwick and Radke 1983, Steward unpublished data).

These results suggest the presence of an evolutionary trap where *P. macdunnoughii* has evolved to recognize specific glucosinolates, such as sinigrin, as oviposition cues, yet *T. arvense* does not support larval development to pupation. *T. arvense* is an invasive mustard from Eurasia first documented in the Rocky Mountain region in the 1800's, and it was predicted by Chew that female butterflies will stop ovipositing on *T. arvense* or if they continue laying eggs on *T. arvense* then variation that allows larvae to reach pupation will be selected for (1977). Forty years later, *P. macdunnoughii* are still observed laying eggs on *T. arvense* with no larval survival, which is resulting in a 3.0% fitness cost to the populations (Nakajima et al. 2013). Exploring the genetic component to this evolutionary trap, there was no indication of variation for larvae to develop on *T. arvense*, but oviposition choice is variable among females and found to be heritable and sex-linked (Boggs et al. unpublished data). A closely related European species, *Pieris napi*, is capable of consuming and developing on *T. arvense*, suggesting the ability to survive on *T. arvense* was lost in *P. macdunnoughii* or gained in *P. napi* since their divergence.

For members of Pieridae, the ability to consume mustards is dependent on the capacity to process glucosinolates that act as herbivore defense. When herbivores destroy leaf tissue glucosinolates are mixed with myrosinases (Wittstock et al. 2004). These enzymes hydrolyze the glucosinolates into toxic isothiocyanates (Wittstock et al. 2004). One means to detoxify the effects of glucosinolates is through glucosinolate sulfatases, which remove the sulfate from the

glucosinolates and prevents them from becoming substrates for the myrosinases (Wheat et al. 2007). The Pieridae evolved a detoxifying enzyme called Nitrile-Specifer Protein (NSP) (Fischer et al. 2008). NSP acts to cause nitriles to be formed instead of isothiocyanates, and the nitriles can then be excreted in the feces (Wittstock et al. 2008). The NSP associated gene exhibits high rates of change supporting adaption and speciation, which may explain the differential success of *P. napi* and *P. macdunnoughii* on *T. arvense* (Fischer et al. 2008).

Research in a similar system with *Alliaria petiolata*, commonly known as garlic mustard and taxonomically similar to *T. arvense* has been studied with *Pieris virginiensis*. As in *T. arvense* and *D. incana*, sinigrin is found in *A. petiolata*. In this system sinigrin is found to not affect larval survival on plants that do not naturally possess it and decrease larval survival when added to plants that do (Davis et al. 2015). This selective negative affect on larvae, suggest a unique metabolic pathway that produces a toxin from sinigrin. Sinigrin was found to break down into thiocyanates, instead of isothiocyanates, and then into hydrogen cyanide (Frisch et al. 2015). This pathway, or a similar one, may be active in *T. arvense* and affect larval survival in species such as *P. macdunnoughii* that may not be adapted to it.

The aims of the proposed research are to determine how *T. arvense* and the chemical sinigrin affect larval feeding behavior. Does *T. arvense* have deterrent or toxic effects on larvae? How does sinigrin added to native mustards without sinigrin, *C. cordiflora*, and with sinigrin, *D. incana*, compare to *T. arvense* with additional sinigrin? It is anticipated that if *T. arvense* has deterrent effects it will cause larvae to consume leaves at a slower rate compared to a *C. cordiflora* that supports larval development. We predict that *C. cordiflora* with added sinigrin will not affect larval consumption rates and survival, because proteins needed to break it into hydrogen cyanide will not be present. When added to *D. incana*, if larval survival decreases then

a similar pathway for sinigrin may exist. However, since *D. incana* is not closely related to *T. arvense*, it is predicted that the pathway will not exist and larval survival will not be affected with the addition of sinigrin. We hypothesize that added sinigrin to *T. arvense* will decrease larval survival, as more hydrogen cyanide can be produced to poison the *P. macdunnoughii* larvae.

Methods

Egg Collection

We collected *P. macdunnoughii* females from the Gothic town site and Rustler Gulch (Figure 1). The butterflies were fed 25% (v/v) honey-water solutions twice daily and kept individually in 6x7 inch cylindrical plastic containers at 27-32°C during the day and 16-22°C during the night with a L16:D8 photoperiod. Each enclosure had a moist paper towel at the bottom to prevent desiccation and single shoots from *T. arvense* and *C. cordifolia* on which to lay their eggs. Plants were maintained in the laboratory with *C. cordifolia* plants collected at Gothic Campsite and *T. arvense* plants collected at various locations around Crested Butte. We used butterflies that laid at least 120 eggs for the field and lab assays. The eggs were chilled in a refrigerator before soaked in a 1% bleach solution for 5 minutes and rinsed twice with water for 3 minutes to prevent vertical transfer of diseases. We placed the eggs in the growth chamber until they hatched into first instar larvae. The larvae were used in three experiments.

Larval Feeding Rates in the Lab

We performed feeding observations in the lab with the *P. macdunnoughii* larvae and *T. arvense* and *C. cordifolia*. We observed larvae from two different populations, the Gothic town site (GT) and Rustler Gulch (RG). We used several families from each population for larvae.

Even numbers of larvae received leaves from *T. arvense* and leaves from *C. cordifolia*. Larvae were placed individually in a 45mm petri dish lined with a moist piece of filter paper and a leaf from the specified plant. Before and after the trial each leaf was photographed with a fixed distance to determine leaf area consumed. We took pictures of the larvae using a dissecting microscope (Leica S6D) at intervals, $t=2$, $t=4$, and $t=6$, and use image software to determine leaf content in the gut. This includes measuring absolute gut content, larval length, and determining the category of gut fullness as either empty, crop, mid, full, or dead (Figure 2). Relative gut content was calculated as absolute gut content divided by larval length. We preserved leaf samples with high and low herbivory in methanol for later chemical analysis after the trial was completed.

Larval Feeding Rates in the Field

For the field experiment, larvae from the same females in the lab assay were used. We placed five larvae on either a *T. arvense* or *C. cordifolia* plant in the field located within the Gothic townsite (Figure 3). The plants were covered with a mesh bag tied off with string. After four hours the larvae and the leaves were collected. The larvae were photographed at the end of the experiment for absolute gut content, larval length, relative gut content, and categorized based on gut fullness.

Larval Feeding Rates with the addition of Sinigrin

The next experimental setup was a manipulative feeding trial with six different treatments. Three different plant species were used: *T. arvense*, *C. cordifolia*, and *D. incana*. The treatments were each plant species painted with water and each species painted with a 0.0564 molar synthetic sinigrin solution so that 50 micromoles of sinigrin were added per gram of leaf

mass. The leaves were allowed to dry before use in the feeding trial. Several families were used from sites including: Rustler's Gulch, South Rustler's Gulch, Gothic Townsite, South Gothic, Avery Campground, Gothic Campground, and Judd Falls Campground (Figure 1). In a petri dish lined with moist filter paper individual larvae were presented with a leaf from one of the six treatments. The leaves were massed and photographed for area at $t=0$ and $t=6$ hours using a fixed distance camera. The larvae were photographed every two hours over a six-hour period and every 24 hours of survival. The photographs were taken using a dissecting scope and using image software the absolute gut content, larval length, relative gut content, and gut fullness category were determined. We kept the larvae for up to five days and at the end we noted survival and took final photographs. During this time the larvae were stored at 25-32°C during the day and 16-22°C during the night with a L16:D8 photoperiod.

Statistics

For all feeding assays gut fullness categories (empty, crop, mid, full, and dead) were analyzed using a multinomial logistic regression (R package *nnet*). The full models were simplified hierarchically using chi-squared tests on residual deviance and Δ AIC values. All multinomial regressions were run using a logit link. For the lab assays the gut content categories at the two-hour, four-hour, and six-hour time points were modeled and tested the three-way interactions of population, treatment, and time (t_h), as well as all component terms. Family was excluded as a predictor to conserve degrees of freedom. For the field the response of the four-hour time point in both the lab and field were modeled and tested the the three-way interaction of treatment, assay, and population, as well as all component terms. For the sinigrin assays, the gut content categories from the two-hour, four-hour, and six-hour time points were modeled and tested using the three-way interaction of sinigrin addition, treatment, and time, as well as all

component terms. Both family and population were excluded as predictors to conserve degrees of freedom.

Results

Laboratory Trials

The resulting model for the laboratory trials, which did not differ significantly from the full model (deviance = 11.188, df = 20, $p = 0.941$, AIC = 1663.442), included the interaction of treatment and time and the main effect of population. Although important to the fit of the model, there was not a significant difference between the RG and GT populations (deviance = 8.350, df = 4, $p = 0.0796$, $\Delta\text{AIC} = -0.350$). The interaction between treatment and time was significant (deviance = 20.624, df = 8, $p = 0.00822$, $\Delta\text{AIC} = -4.624$).

In Figure 4, the change in gut content category across time points is visible, but this change is different between the two treatments. The larvae on *C. cordifolia* quickly filled their guts with over 75% of larvae full at the six-hour time point. For the *T. arvense* treatment larvae mostly stayed within the empty, crop, or mid categories with less than 25% ever reaching a full gut at the six-hour time point.

Field Trials

The final model for the field trials did not differ significantly from the full model (deviance = 27.535, df = 20, $p = 0.121$, $\Delta\text{AIC} = 12.465$). *T. arvense* and *C. cordifolia* treatments differed (deviance = 153.401, df = 4, $p < 0.0001$, $\Delta\text{AIC} = -145.4009$), as did the field and lab assays (deviance = 77.700, df = 4, $p < 0.0001$, $\Delta\text{AIC} = -69.700$). This main effect of assay seems to have been largely driven by the larvae eating *C. cordifolia* in the lab assay, more than 50% of which reached full guts for both populations, while fewer than 25% did the same on *C. cordifolia*

in the field assay (Figure 5). However, there was no significant interaction between assay and treatment (deviance = 5.07797, df = 4, $p = 0.2793905$, $\Delta AIC = 2.92203$). The larvae on *T. arvense* ate less, never surpassing 20% on full guts.

Sinigrin Trials

For the sinigrin trials the final model, which did not differ significantly from the full model (deviance = 28.708, df = 40, $p = 0.908$, $AIC = 1045.833$, $\Delta AIC = 51.292$), included a significant interaction between plant treatment and sinigrin addition (deviance = 20.646, df = 8, $p = 0.00815$, $\Delta AIC = -4.646$). The main effect of time was also significant (deviance = 38.771, df = 8, $p < 0.0001$, $\Delta AIC = -22.771$). Unlike the lab assay, the interaction of treatment and time was not significant (deviance = 16.643, df = 16, $p = 0.409$, $\Delta AIC = 15.356$).

Gut fullness category differed between plant treatments, and this difference was enhanced by the addition of sinigrin to the leaves, where more larval death occurred. The addition of sinigrin had the least effect on larvae consuming *D. incana*, and the largest effect on larvae consuming *C. cordifolia*. Larvae consuming *T. arvense* treated with sinigrin were most likely to remain empty throughout the assay.

Discussion

The objective of this research was to determine how *T. arvense* affects the feeding behavior of first instar *P. macdunnoughii* larvae. From the laboratory feeding assays, it is not clear that *T. arvense* kills the larvae through acting as a deterrent and causing starvation or as a toxin. From the data, it is evident that the larvae do not consume as much *T. arvense* as *C. cordifolia*, suggesting there are deterrent properties within the leaves. This is consistent with recent studies that determine deterrent properties based on the reduced herbivory compared to

standard host plants (Ulrichs et al. 2008). However, since over 50% of the larvae end up consuming some *T. arvense*, the larvae are not only dying of starvation.

The marginal non-significance between butterfly populations and their feeding behaviors on *T. arvense* and *C. cordifolia* shows that the feeding behavior was not different between the two populations. This observation is interesting considering that *T. arvense* is present around the Gothic townsite where the GT butterflies were collected, while Rustler's Gulch does not have *T. arvense* present. Based on the predictions of escaping the evolutionary trap and adapting to eat *T. arvense*, it would be expected that GT larvae would exhibit more feeding behavior towards *T. arvense*. The lack of difference suggests that no selection for larval feeding is occurring.

Visualizing the data another way, the feeding behavior appears to vary among the different families (Appendix, Figure 7). The variation for feeding on *T. arvense* fluctuates from not eating at all as a family to eating about mid gut as a family. The presence of such variation suggests that there is some component of heritability for larval feeding, which currently does not include the capability to feed on *T. arvense*. However, the variation provides the opportunity for specific genes to be selected for that allow larvae to avoid or feed on *T. arvense*.

Comparing the laboratory results at the four-hour time point to the field assay results, the assays were significantly different. This difference is most likely the result of greater feeding activity occurring in the *C. cordifolia* treatment in the laboratory setting with over 50% having full guts, while the field assays only reached around 25% full guts. One factor that may have contributed to this difference is temperature, which was regulated for the laboratory trials around 30°C. In the field trials the temperature ranged from 15-30°C and on Field Assay Day 2 the temperature never exceeded 16°C. On this day most larval guts were empty and for this reason we excluded it from the analyses. The lower temperatures, which were also decreased for being

inside a bag and on the underside of the leaves, most likely decreased larval metabolism and feeding. Furthermore, even though the assays are different the observed trends are the same with larger larvae proportions consuming *C. cordifolia* than *T. arvense*.

For the sinigrin assays, sinigrin has a noticeable effect on all plant treatments. For *T. arvense* the proportion of larvae with empty guts rises from around 25% on water treatments to near 50% on sinigrin treatments at all time points. This shows that the addition of sinigrin makes the larvae less likely to consume *T. arvense*, which suggests that sinigrin acts as a deterrent. This is further supported by larval feeding behavior on *C. cordifolia* and *D. incana*. For *C. cordifolia*, larvae went from over 50% with full guts on the water treatment at the six-hour time point to below 25% with full guts on the sinigrin treatment. The proportion with empty guts remained above 25% for the sinigrin treatment, while water treatments never surpassed 25%. These behaviors are strongly indicative of a deterrent property. Finally, *D. incana* was the least negatively affected, but at all time points the number of larvae with full guts in the sinigrin treatment was approximately half of the number with full guts in the water treatments. This suggests that larval feeding was slowed, but *D. incana* was still largely consumed. Though there are clearly deterrent properties from the addition of sinigrin there are also some toxic properties. In comparison to the water treatments the sinigrin treatments showed higher proportions of death, which for all plant species was around 25% at the six-hour time point.

The different responses to *D. incana* and *C. cordifolia* with added sinigrin suggests that other mechanisms are involved for deterrence, as it was a large deterrent for *C. cordifolia* and less so for *D. incana*. As sinigrin is naturally found in *D. incana* and does not affect feeding as it does for *T. arvense* this suggests a different metabolic breakdown of sinigrin that does not result in isothiocyanates or hydrogen cyanide. Though sinigrin is not found *C. cordifolia* a more

harmful break down of sinigrin may occur with the different enzymes present in *C. cordifolia* to cause the increased deterrence. This interaction requires more investigation to what other chemicals may be involved in larval death on *T. arvense* as well as the chemical pathway that sinigrin takes with enzymes from the different plants.

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Tables and Figures

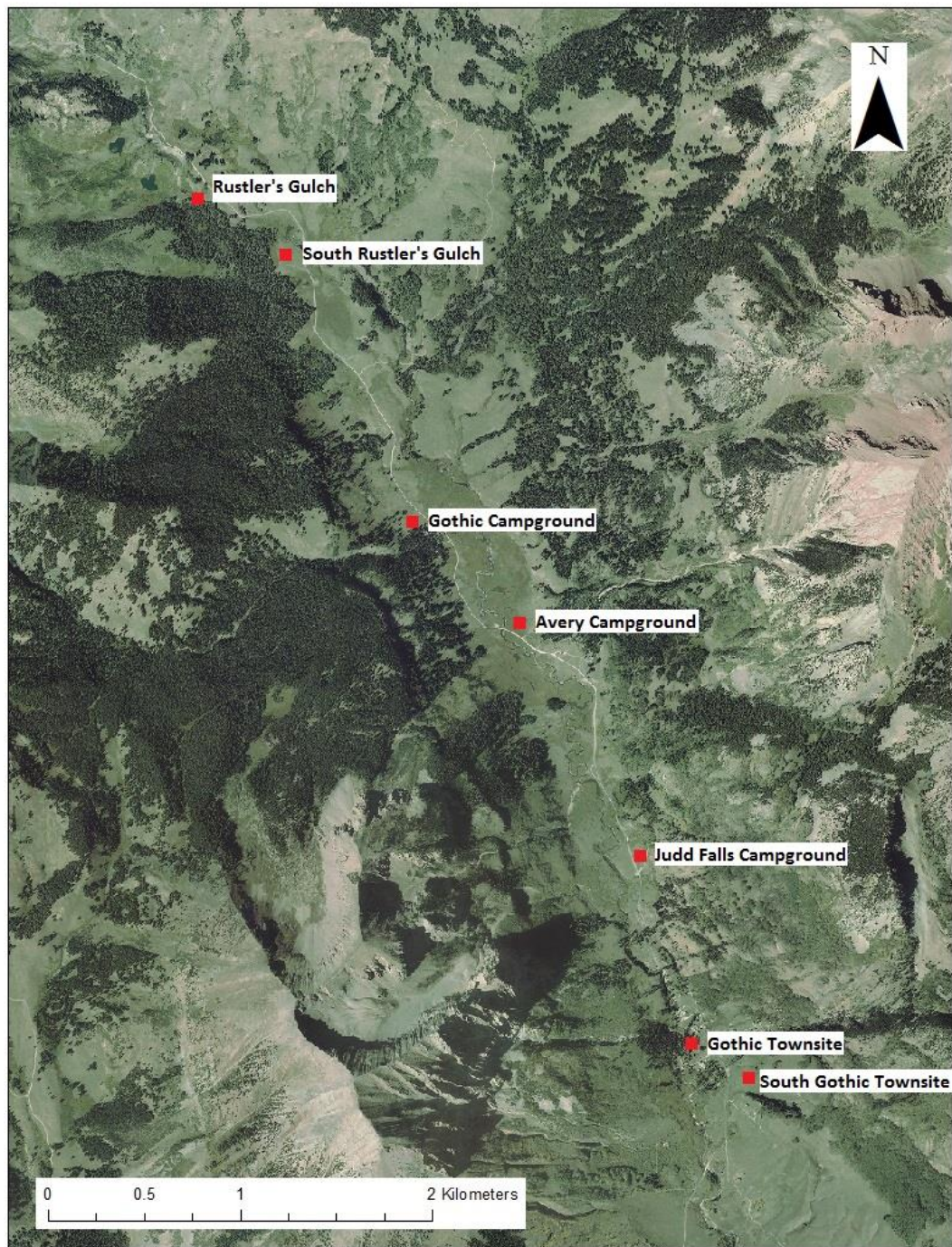


Figure 1. *Pieris macdunnoughii* females were collected in the Gunnison Valley at Rustler's Gulch, South Rustler's Gulch, Gothic Campground, Avery Campground, Judd Falls Campground, Gothic Townsite, and South Gothic Townsite.



Figure 2. Examples of different gut content categories: a – empty, b – crop, c – mid, and d – full.



Figure 3. Location of field assays with different plots established for the two treatments with either *Thlaspi arvense* or *Cardamine cordifolia*.

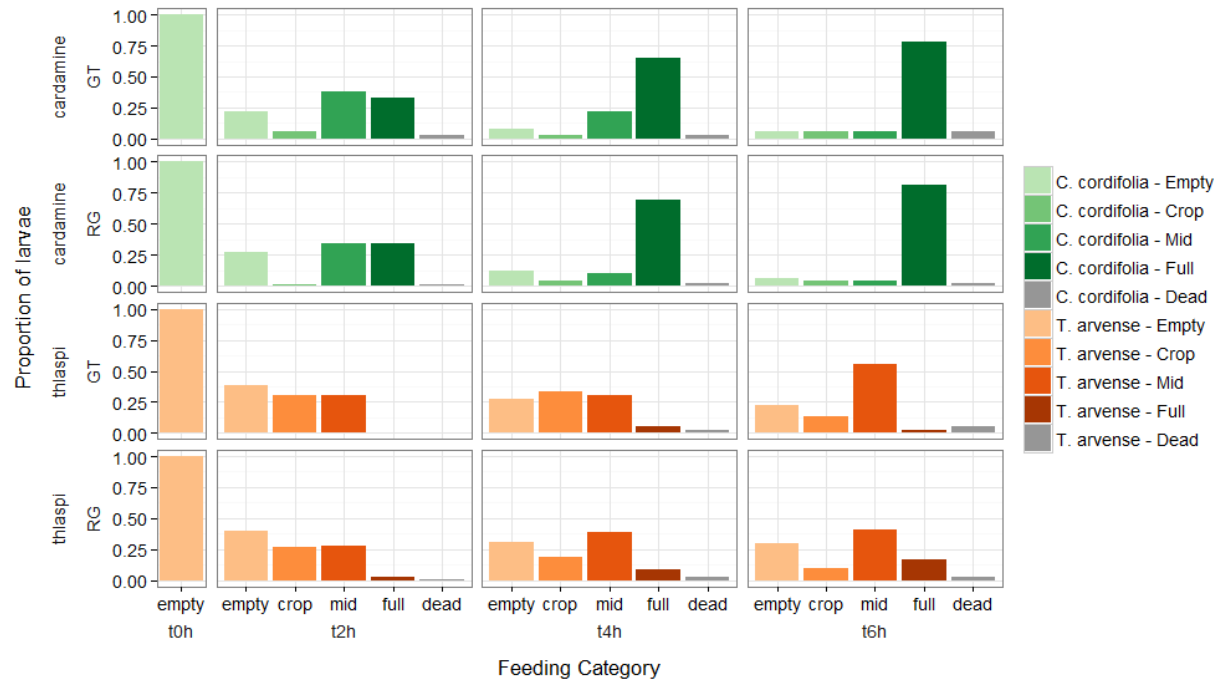


Figure 4. Proportion of first instar larvae in each gut content category-- ‘empty’, ‘crop’, ‘mid’, ‘full’ or ‘dead’ -- across time. Larvae from GT and RG populations were fed *C. cordifolia* (green shades) and *T. arvense* (orange shades) over six hours, with gut measurements taken every two hours.

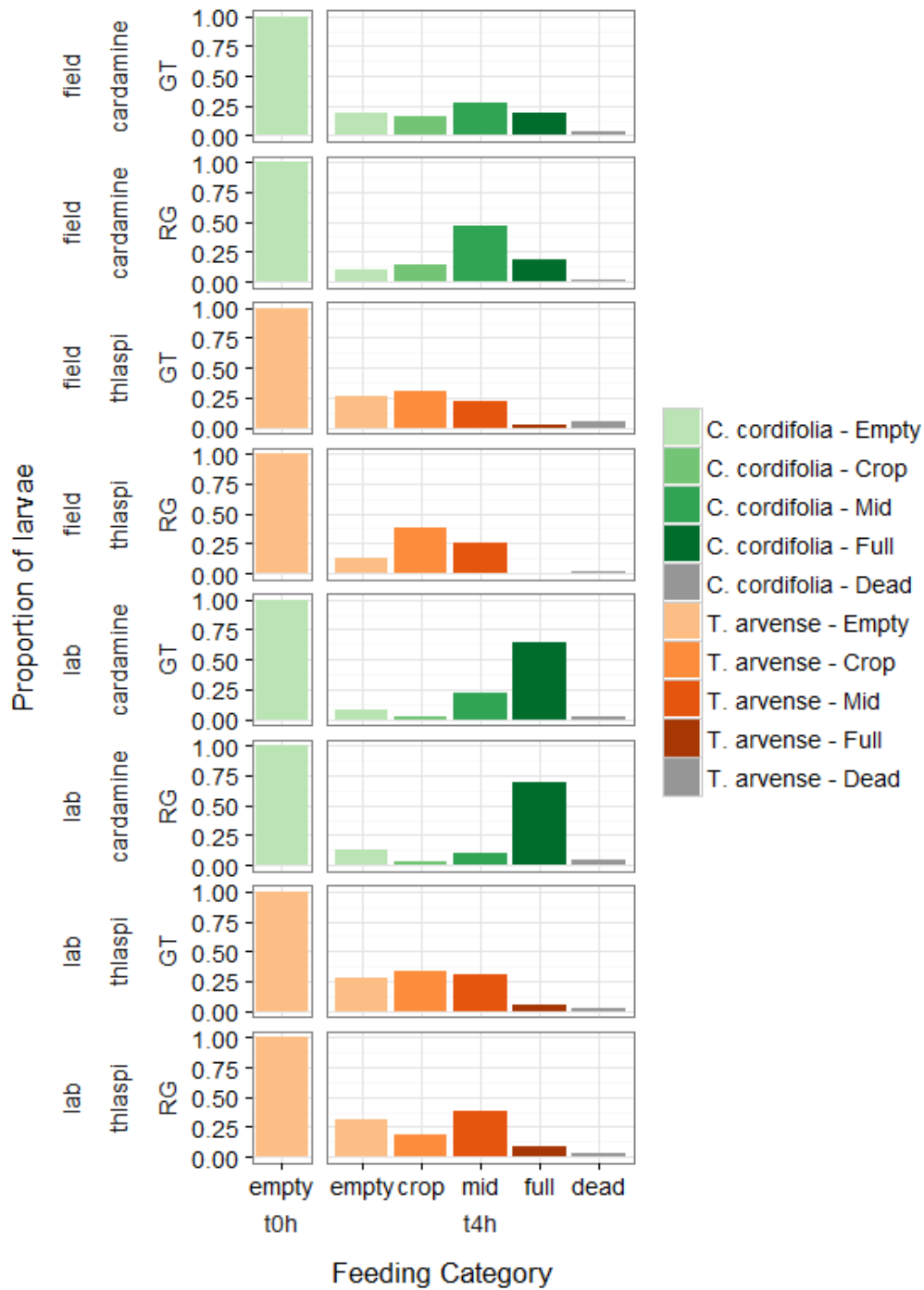


Figure 5. Proportion of first instar larvae in each gut content category-- ‘empty’, ‘crop’, ‘mid’, ‘full’ or ‘dead’ -- across time. Larvae from GT and RG populations were fed *C. cordifolia* (green shades) and *T. arvensis* (orange shades), either in the form of cut leaves in the lab assay or whole stems in the field assay. Larval gut content was assessed at 4 hours.

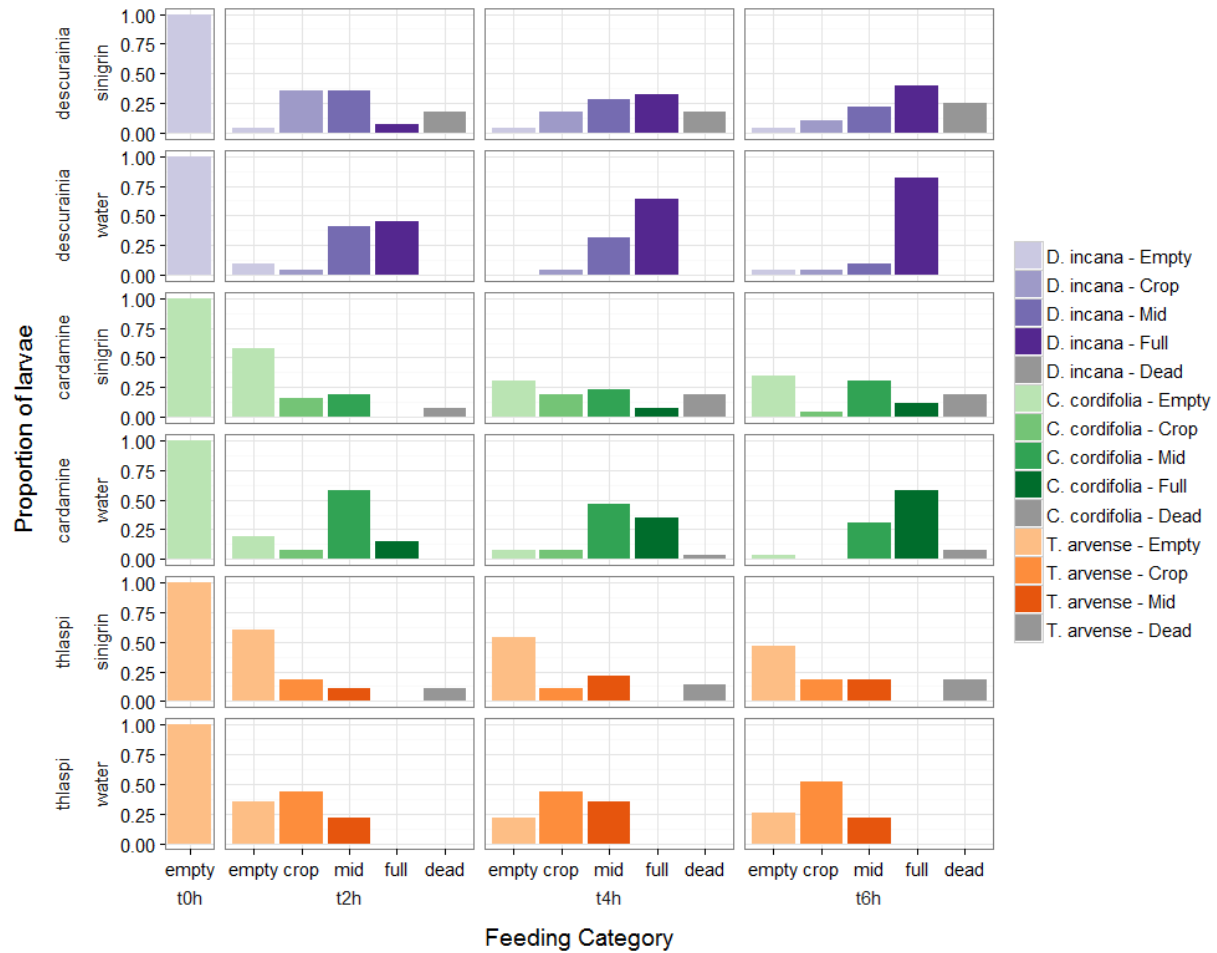


Figure 6. First instar larval gut content by category. The proportion of larvae in each family falling into the gut content categories ‘empty’, ‘crop’, ‘mid’, ‘full’ or ‘dead’. Larvae were fed *D. incana* (purple shades), *C. cordifolia* (green shades) and *T. arvensis* (orange shades) over six hours, with gut measurements taken every two hours.

Appendix:

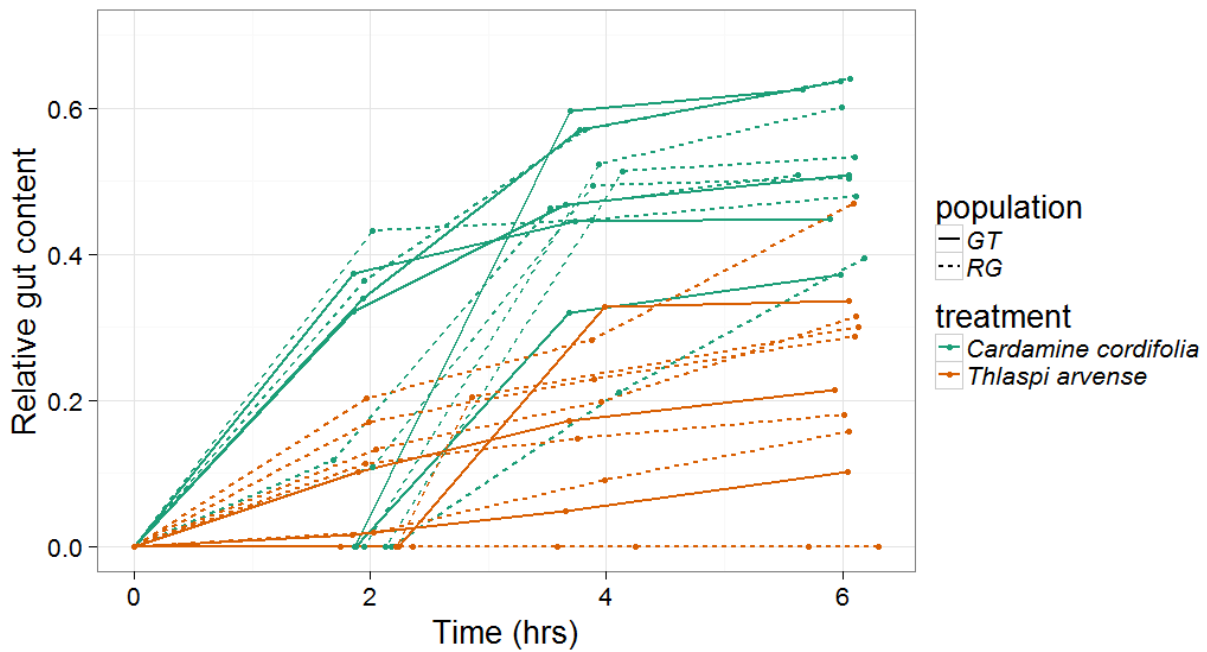


Figure 7: Mean relative gut content by family, population, and treatment over six hours. Larvae from GT (solid) and RG (dashed) were fed with *T. arvense* (orange) or *C. cordifolia* (green).