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# The effect of sideroxylonal-A on feeding of steelblue sawfly, *Perga affinis affinis* Kirby (Hymenoptera : Pergidae), larvae

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**Abstract.** Plant secondary metabolites can affect insect feeding but responses are species-specific. Sideroxylonal-A (a formylated phloroglucinol) has been shown to inhibit feeding in several vertebrate and invertebrate herbivores. To investigate whether sideroxylonal-A affected feeding in sawfly larvae, *Perga affinis affinis*, we fed larvae eucalypt leaves containing various concentrations of sideroxylonal-A, and measured frass production as an indicator of consumption. We found that: (1) at least 80% of the sideroxylonal-A ingested by larvae was stored in the diverticulum; (2) less than 1% was excreted in frass; (3) feeding was unaffected by the concentration of sideroxylonal-A; and (4) larvae produced more frass on natal host leaves than on non-natal host leaves.

**Additional keywords:** feeding deterrent, formylated phloroglucinols, insect herbivory.

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## Introduction

Eucalypts (Myrtaceae) produce high concentrations of plant secondary metabolites (PSMs) that potentially reduce insect herbivory (Ohmart and Edwards 1991). The predominant eucalypt secondary metabolites are phenolics and terpenes that have both pre- and postingestive effects in insects (Martin *et al*. 1987; Felton and Duffey 1991; Appel 1993, 1994), although other secondary compounds are present (Santos *et al*. 2019).

Formylated phloroglucinol compounds (FPCs) are a group of PSMs that are present in several eucalypt species in the informal subgenus *Symphomyrtus*. Sideroxylonal-A is an FPC that is present in varying concentrations in the foliage of several eucalypts, including *E. melliodora*, *E. sideroxylon*, and *E. polyanthemos*, may be colocalised with terpene oils such as cineole (Lawler *et al*. 1999; Goodger *et al*. 2016), and has been shown to inhibit feeding in both marsupials and insects (Lawler *et al*. 1998; Henery *et al*. 2008; Matsuki *et al*. 2011*a*, 2011*b*). However, many insects are known to feed on eucalypts potentially containing sideroxylonal with no evidence of detrimental effects (Ohmart and Edwards 1991). One such species is the Australian steelblue sawfly, *Perga affinis affinis* Kirby (Hymenoptera: Symphyta: Pergidae).

Larval sawfly morphology has been described (Maxwell 1955; Tait 1962), but little is known of the physiology of Australian sawflies and their processes for consuming the foliage of *Eucalyptus* species that contain relatively high concentrations of PSMs. Larvae of *P. affinis affinis* form large semisocial groups that cluster together during the day and forage at night (Carne 1962). During feeding bouts, several individuals forage together by following 2–3 leader animals away from the main cluster (Weinstein and Maelzer 1997; Hodgkin *et al*. 2014, 2017). Larvae will feed for up to 8 h, then will follow the leading animals back to the main cluster. The main cluster can be as many as 100 individuals, and feeding appears to occur nightly. However, the host plant is chosen by the ovipositing adult female, and larvae will normally feed on the same plant from hatching until they are ready to pupate (Carne 1962).

High concentrations of PSMs may cause sawflies to reduce feeding, as occurs in some beetles and caterpillars (Henery *et al*. 2008; Matsuki *et al*. 2011*a*, 2011*b*). Rather than a reduction in feeding occurring, feeding could be maintained or even increased if an unknown proportion of ingested PSMs are stored in the pharyngeal diverticulum (Maxwell 1955; Schmidt *et al*. 2000, 2010). If the latter, these compounds could be used when regurgitated in defence against predators (Schmidt *et al*. 2000). The regurgitant has been shown to contain the same oils that are present in leaves of the host plant (Morrow *et al*. 1976). Oil glands that contain the PSMs are separated from the leaves during feeding using mandibular brushes and transferred into the diverticula (Schmidt *et al*. 2000, 2010; Schmidt and Walter 2011). However, it is not known if other secondary plant compounds are also stored in the diverticula.

This study examined the extent to which *P. affinis affinis* larvae are capable of feeding on leaves containing varying levels of sideroxylonal-A, given that it is known to affect feeding in other species. We used frass production as a

 

measure of food consumption. To understand how *P. affinis affinis* copes with PSMs like sideroxylonal-A, we investigated the presence of siderxylonal-A in the contents of the diverticula and frass, as well as whether the compound is toxic when present in the hæmolymph. The contents of regurgitant and gut were also compared using microscopy to determine how regurgitant and digesta differ in their appearance and whether oil glands are observable as that may indicate how PSMs may be moved along with oils into diverticula. The gut was also examined as damage to the peritrophic membrane has been reported in beetle larvae that consumed eucalypts (Henery *et al*. 2008). Using the information from sideroxylonal concentration, feeding and excretion rate and volume of the diverticula, a budget of sideroxylonal-A input, storage, and excretion was estimated to help understand how *P. affinis affinis*, may cope with consuming eucalypt PSMs.

## Methods and materials

### General insect care

Sawfly larvae were collected from the field for all experiments and returned to the laboratory. Animals were kept at a constant temperature during experiments (16 ± 0.5°C) and given access to cut eucalyptus branches. By allowing the animals to forage for the food, as occurs in the field, the animals gained weight and stayed healthy. To keep the leaves moist, the branches were placed in flasks containing 100 mL water, with aluminium foil covering the flask top to prevent larvae and frass from falling into the water. Cages were wooden with flyscreen sides (25 × 28 cm, 31 cm deep). Two large holes in the base permitted cups to be inserted, and one of the cups in each cage contained the conical flask of water into which branches were placed (Carne 1962). The limited space within the constant temperature room meant that only one group of feeding studies could be done, so that replication had to be extended over time.

### Contents of gut

For whole-animal dissection, fifth-instar larvae ($n$ = 5) were placed in a freezer at –20°C for 15 min, then removed and immediately immersed in cold (4°C) 70% ethanol. Insects were dissected within two days using a dissecting microscope (Wild M8), as any longer in ethanol resulted in loss of material from the diverticula. Images of the dissections were captured using a Nikon ShuttlePix 400v microscope and camera.

Sections of the digestive system were made from larvae used in the feeding studies that were embedded in wax. Briefly, larvae ($n$ = 3) were cooled (at 4°C) for 30 min, dissected open and the digestive system, including diverticula, was removed. The tissues were immersed in Bouin's solution (48 h) under vacuum, washed with 70% ethanol to remove the fixative, and then dehydrated in ethanol (70–100%). Dehydrated tissues were infiltrated with paraffin wax under vacuum (3 × 2 h) and then embedded in wax. Sections (7 µm) were cut on a rotary microtome (Leica RM2125), with sections placed on a slide and immersed in hot water (55°C) containing gelatine to ensure tissue adherence. Slides with sections were stained using periodic acid–Schiff's reagent–alcian blue to differentiate the tissues. Sections were coated with Depex® and protected using coverslips.

### Regurgitant

Stimulation of live fifth-instar larvae ($n$ = 3) collected from trees not used for feeding studies resulted in some regurgitation of diverticula material that was collected using glass Pasteur pipettes and deposited directly onto slides. The regurgitant was covered with a coverslip. Images for both sections and regurgitant were recorded using a Leica MC 120 HD camera mounted on a Leica DMLB microscope and collected (Dell Latitude E6540).

### Preliminary feeding experiments

Directly measuring the quantity of food that was eaten by the larvae each day was not feasible, as larval feeding ceased in response to disturbances, sometimes for long periods. Preliminary feeding experiments were conducted to determine whether frass production was a reliable method of indirectly determining the rate of feeding. This involved:

(1) determining passage time (the time between feeding and frass production);
(2) determining the relationship between feeding and frass production; and
(3) determining the relationship between leaf area and mass.

Passage time was measured by offering larvae isolated leaves of their host plant (*Eucalyptus melliodora*) that had been dyed with azorubin, and frass examined for signs of the dye at 6-h intervals using a dissecting microscope (Wild M8). The presence of dye in the frass indicated that food took 24–36 h to pass through the larvae. Therefore, for subsequent studies, frass was collected 24 h after feeding.

For determining the relationship between feeding and frass production, 22 larvae were taken from the host plant (*E. melliodora*) and separated into 11 pairs. Pairs were used rather than single larvae, as Fletcher (2007) indicates that single larvae tap for long periods and single larvae have been shown to not always feed (Schmidt *et al*. 2010). Pairs were kept in plastic jars (5.5 cm high, 4.2 cm diameter). Larvae were not fed on the first day. For four consecutive days, larvae were offered fresh leaves of known mass from the host tree and frass and unconsumed leaf-matter were collected. On the sixth day, larvae were preserved in 70% ethanol and weighed. The frass and leaves were oven-dried at 60°C for one week. The dried frass and leaves were weighed, and leaf surface area was measured using a leaf area planimeter (Paton Electronic Planimeter). Repeated-measures regression was used to determine the relationship between food consumption and frass production for the 11 pairs of larvae over the four days of the experiment (day was the repeated measure).

The relationship between leaf area and leaf mass was obtained through weighing and measuring leaf areas of 150 fresh leaves. Regression analysis was used to determine the relationship between fresh leaf mass and fresh leaf area. Because the leaves shrank slightly with dehydration over the feeding period, a correction for shrinkage was done with control branches that were kept in the same conditions.

Calculated fresh leaf area consumed by the larvae was regressed against the quantity of frass produced by the larvae, with fresh mass converted to dry mass using control leaves that were allowed to dry during the feeding studies.

### *Feeding studies*

Three replicate feeding studies were performed using three separate colonies collected from trees that varied in the leaf concentration of sideroxylonal-A. The host plants and their sideroxylonal-A concentration (mg $g^{-1}$ dry mass) were *E. macrorhyncha* (0 mg $g^{-1}$), *E. melliodora* (22.2 mg $g^{-1}$), and *E. melliodora* (39.5 mg $g^{-1}$).

Each feeding study ran for 20 days from the day that the colonies were collected. The three feeding studies were performed sequentially, the first in April, the second in July, and the third in August. As a result of the timing of the studies, some variation in age structure of each colony was unavoidable, although with the first two feeding studies, mean mass of the individuals used was similar. Each colony was separated into three clusters (7–8 individuals each of approximately same total mass) that were kept in separate cages within the same room. The cages were arbitrarily termed A, B and C, and their positions during the experiment were randomly determined. A removable tray was designed to rest on the floor of each cage to collect frass, and was emptied every morning at 0900 hours. The experimental protocol was a Latin square arrangement, such that each cluster would undergo three treatments over the course of the feeding study, and at any time during the study there would be one cluster in each treatment (Table 1).

The treatments were feeding on leaves of three different trees (not their host plant) with varying concentrations of sideroxylonal-A. Initially, clusters were offered branches with leaves from the host plant for five days, allowing larvae to adapt to the laboratory conditions and resume a regular feeding pattern. On the morning of the third day, the branches with leaves were replaced with fresh branches and leaves from the same plant, and from then on branches with leaves were changed every three days to ensure leaf moisture remained high. After the initial five days of feeding on the host plant, clusters of larvae were offered branches with leaves from the three trees varying in content of sideroxylonal-A.

**Table 1. The experimental design used in this study**

Feeding studies were organised to a partial Latin square design, with natal leaves acting as a control and each group feeding on all treatment diets in the course of the experiment (only the second and third natal leaf feeding was used for control as animals were not completely settled during natal leaf feeding on Days 1–5)

| Time in experiment | Cage A | Cage B | Cage C |
|---|---|---|---|
| Days 1–5 | Natal leaves | Natal leaves | Natal leaves |
| Days 6–8 | Treatment 1 | Treatment 2 | Treatment 3 |
| Days 9–11 | Natal leaves | Natal leaves | Natal leaves |
| Days 12–14 | Treatment 2 | Treatment 3 | Treatment 1 |
| Days 15–17 | Natal leaves | Natal leaves | Natal leaves |
| Days 18–20 | Treatment 3 | Treatment 1 | Treatment 2 |

Trees were selected from a group of *E. melliodora* with known sideroxylonal concentrations at Mulligan's Flat Nature Reserve, Australian Capital Territory (35°10′S, 149°10′E). In the first two experiments two medium concentrations (both 18 mg $g^{-1}$) of sideroxylonal-A were offered as Tree 1 and Tree 2 and one high concentration (30 mg $g^{-1}$) as Tree 3. In the third study, leaf concentrations in one tree had decreased and the leaf concentrations were 13, 18 and 30 mg $g^{-1}$ sideroxylonal-A respectively. Between each three-day treatment, clusters of larvae were offered leaves from their natal host plant for three days. The data from these three-day periods were used as control data for the second two treatment periods. Data from the initial five-day host plant period were not used in statistical analyses, as larval behaviour in this period appeared erratic, and feeding was considered to be atypical. There was thus no control data for the first three-day treatment period.

### *Injections of sideroxylonal*

We examined the effect on larvae of absorbing sideroxylonal in the midgut by injecting sideroxylonal-A solutions into the hæmocoel. Carrier solution, volume injected, and presence of sideroxylonal were the independent variables and partial and full recovery times as the dependent variables in a multiple analysis of variance.

Sideroxylonal-A is poorly soluble in aquaeous solutions, so three different carrier solutions were used:

(1) phosphate-buffered saline (PBS, pH 8.0);
(2) a 9 : 1 ratio of PBS and methanol in which sideroxylonal-A had been dissolved before mixing with PBS; and
(3) pure methanol in which sideroxylonal-A had been dissolved.

In Solutions 2 and 3, all sideroxylonal-A was dissolved, but in Solution 1, most sideroxylonal-A was in suspension. The concentrations of sideroxylonal in the three solutions were 17, 1.7 and 17 mg $mL^{-1}$, respectively, as determined by vacuum evaporation (Jouan RC-10).

For each solution, various volumes were injected (1, 2.5 or 5 μL) to ascertain whether volume injected affected recovery.

For injection, individual larvae were injected with either the carrier solution with sideroxylonal (treatment) or carrier solution alone (control).

The larvae were removed from colonies that were feeding on *E. melliodora*, *E. mannifera* or *E. blakelyi* and held in the laboratory for 24 h before experiment, and then the larvae were placed under carbon dioxide anaesthesia before injection. Injection was done in abdominal segments 7–9 using a 25-μL syringe with a 0.362-mm (28 gauge) needle (Hamilton) with injection volume controlled using a syringe microburet (Aloe SB2). All experiments were done at 22°C.

After injection, the larvae were placed in plastic containers with a twig under a 75-W light globe and their time to partial and full recovery measured. Gripping the twig was used as an indication of partial recovery, and tapping, feeding, or coordinated movement was assumed to be full recovery.

### *Laboratory analysis for sideroxylonal-A budget*

To determine the proportion of ingested sideroxylonal-A that was metabolised in the larval gut during feeding, we calculated

the amount of sideroxylonal-A ingested, the proportion stored in the pharyngeal diverticulum, and measured the proportion excreted in frass for all larvae from the feeding study. The proportions of sideroxylonal-A stored and excreted in frass were calculated and subtracted from the quantity of ingested sideroxylonal-A, leaving a portion that was unaccounted for. This quantity was assumed to be the quantity of sideroxylonal-A that had been metabolised by the larval gut.

After each experiment ended, larvae were preserved and weighed. Leaf samples were freeze-dried or air-dried at 30°C and ground for near-infrared reflectance spectroscopy (NIRS) (Perstorp Analytical NIRSystems 6500; data processed using Scan.exe 04.00, Infrasoft International, Inc.) to measure the concentration (mg $g^{-1}$ dry mass) of sideroxylonal-A (Wallis and Foley 2003).

The amount of sideroxylonal-A ingested was calculated by multiplying the estimated mass consumed by the sideroxylonal-A concentration of the leaves consumed. Sideroxylonal-A concentrations (mg $g^{-1}$ dry mass) in the leaves of trees used in the feeding study were determined by NIR spectral analysis. The regression between leaf area consumed and quantity of frass produced was used to estimate the leaf area consumed by the larvae during the feeding study. Leaf area was translated into dry mass consumed from the regression between fresh leaf mass and fresh leaf area.

To estimate the proportion of sideroxylonal-A stored in the pharyngeal diverticulum, we ascertained the concentration of sideroxylonal-A in the regurgitant, estimated the volume of fluid in the diverticulum, and converted that volume to mass.

The concentration of sideroxylonal-A in the regurgitant from the pharyngeal diverticulum was determined for clusters feeding on five separate trees of known concentration of sideroxylonal-A. Known masses of regurgitant were collected from each cluster and dissolved in methanol (high performance liquid chromatography (HPLC)–grade) and analysed for sideroxylonal-A using HPLC according to the same technique used for plant material.

Diverticulum volume was estimated by $^{14}$C-inulin dilution (Wharton *et al*. 1965) for individual larvae by encouraging regurgitation, adding a 2-µL aquaeous $^{14}$C-inulin standard (7.4 kBq $\mu L^{-1}$) using a microcapillary tube (Drummond) to the regurgitated solution, and then letting the larvae reabsorb the regurgitant. After 1 h, the animals were again stimulated to regurgitate and a 2-µL sample taken using microcapillary tube, immediately added to scintillation fluid and $^{14}$C-inulin in this sample determined using a scintillation counter (Beckman). Saline standards of increasing volumes in which $^{14}$C-inulin was added were treated in the same way and volume of diverticula determined by comparison with standards by comparing the β counts. Volume measured for individual larvae was then regressed against body mass, and the regression equation was used to estimate diverticular volumes of animals used in the feeding studies.

For calculation of mass of diverticular fluid, the estimated volumes of diverticular fluid were converted to mass of material in diverticula using the density (1.91 mg $\mu L^{-1}$) of the regurgitant measured by collecting regurgitant in 2-µL microcapillary tubes, weighing the tubes with regurgitant, then drying in a 55°C oven and reweighing the tubes. That total mass was then multiplied by the concentration of sideroxylonal-A in the regurgitant in order to determine the mass of sideroxylonal-A in the diverticulum.

As frass had not previously been examined for concentration of sideroxylonal-A, NIRS could not be used without first doing a correlation analysis with HPLC, so concentrations were directly measured with this technique. Dried frass was weighed, ground and analysed for sideroxylonal-A using the HPLC procedures as previously described (Wallis *et al*. 2003; Wallis and Foley 2005).

### *Statistical analysis*

For the preliminary feeding experiment, a regression between estimated leaf area consumed and frass produced was performed using repeated-measures analysis (11 pairs × 4 days) (JMP 6.0), as this method permits comparison of frass production with leaf consumption over time by the pairs of larvae, with pairs included as a random variable. Statistics for frass production from the feeding studies used log-transformed data as the inverse log of frass produced per individual over each three-day period. An analysis of variance (ANOVA) based around the Latin square design was used to examine variation in frass production amongst the treatments. To compare frass production between the natal and non-natal plants, restricted maximum-likelihood (REML) method was used because of the unbalanced design (no initial period when larvae were feeding on host plant) with a change in deviance (CID) used to determine significant effects, as a CID response approximates a Chi-square distribution. These analyses were performed using Genstat 5 release 4.1 (Rothamsted Experimental Station). Regression and MANOVA (multiple analyses of variance) analyses were performed with JMP 13 (SAS).

## Results

### *Digestive system structure and contents*

The triangular-shaped foregut sits on top of the diverticula (Fig. 1*a*), with the tracheated, paired diverticula sitting just below (Fig. 1*b*). Fat body is present surrounding both these tissues. No foliar oil glands appear to be present in the food, although other plant material (such as epidermal cells connected to palisade parenchyma) is clearly present in various stages of digestion at the beginning of the midgut (Fig. 1*c*). Higher magnification of the peritrophic membrane lining the midgut showed an intact and normal membrane in all larvae (Fig. 1*d*) The regurgitant seems to contain various amorphous structures (Fig. 1*e*) with recognisable plant cells only being present occasionally within small components of regurgitant.

### *Relationship between leaf area and leaf mass*

Leaf area ($mm^2$) increased with either leaf fresh weight (g) ($F_{1,148}$ = 1139, $P < 0.001$, $r^2$ = 0.88, area = 76.1 + 2612 (±77.4) mass), or dry mass (g) ($F_{1,148}$ = 1844, $P < 0.001$, $r^2$ = 0.96, area = 52.5 + 4071 (±94.8) mass).

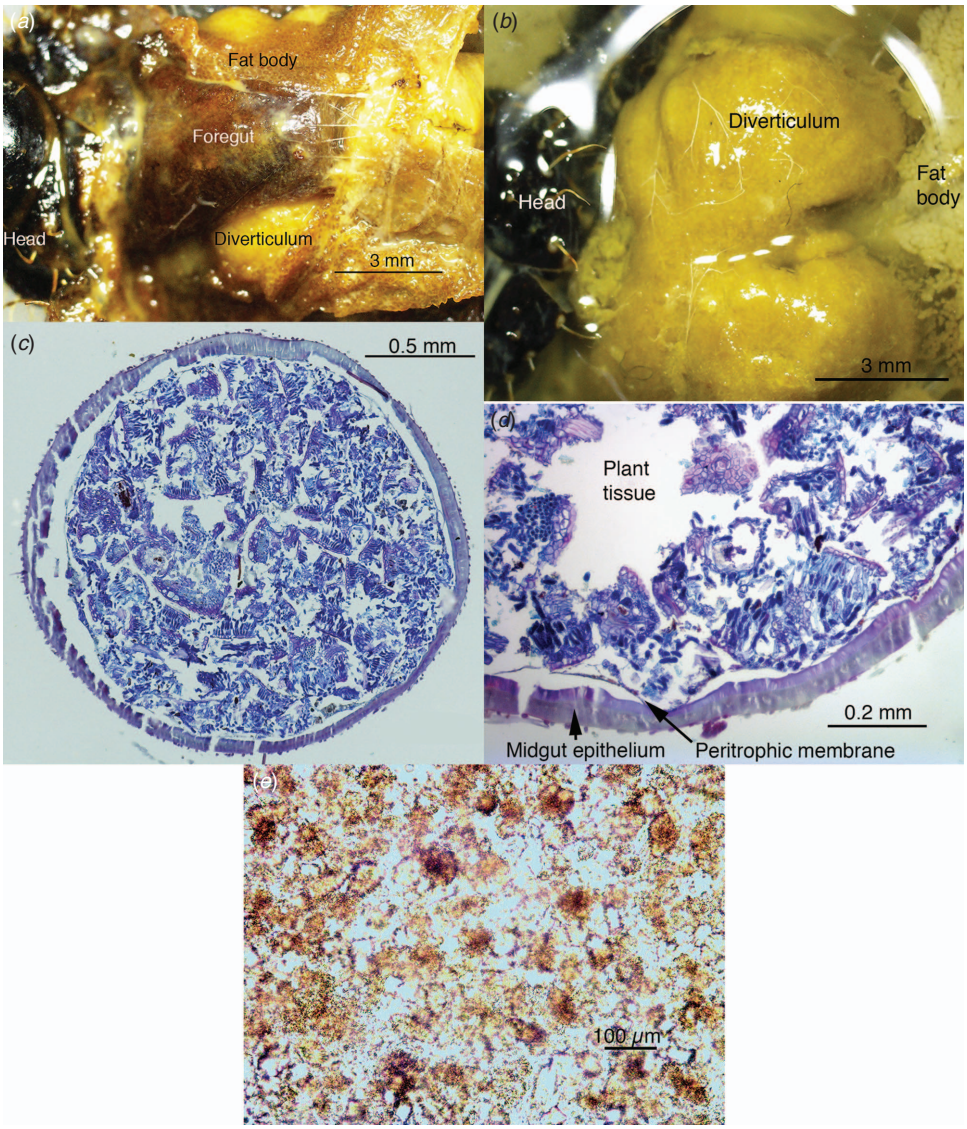


**Fig. 1.** Micrographs of the foregut, diverticula, internal midgut and regurgitant of *Perga affinis affinis* last-instar larva. (*a*) Organisation of foregut and diverticulum in larva; (*b*) diverticulum showing size and expansion as a result of storage of ingested material; (*c*) midgut region showing identifiable plant material; (*d*) higher magnification of midgut region to show food, peritrophic membrane around food and midgut epithelium; (*e*) regurgitant from diverticulum showing various amorphous structures.

### *Relationship between frass production and leaf area consumed*

Fresh leaf area consumed by the larvae in the validation studies was calculated from mass consumed. The mass of frass (mg) produced increased with calculated leaf area consumed ($mm^2$) (dry mass frass produced (mg) = 10.3 (±14.4) + 0.04 (±0.01) leaf area ($mm^2$); $F_{1,43} = 27.6$, $P < 0.001$, $r^2 = 0.84$, repeated-measures REML method), and validated the measure of frass production as a method for determining rate of feeding.

### *Feeding studies*

Larvae from the first two feeding studies were between third and fifth instars (Table 2), and all larvae from the third feeding study were in their final larval instar (Table 2). Because of the size differences, a regression of body mass against diverticulum volume was necessary to estimate the proportion

**Table 2. Mass and head capsule width of larvae collected in April, July and August to indicate the size difference among the three colonies used in the feeding studies**

Data shown are mean ± s.e. (*n*)

| Month | Mass (g) | Head capsule width (mm) |
|---|---|---|
| April | 0.75 ± 0.037 (21) | 3.82 ± 0.085 (21) |
| July | 0.74 ± 0.044 (22) | 4.18 ± 0.066 (22) |
| August | 1.52 ± 0.076 (24) | 4.59 ± 0.041(24) |

of sideroxylonal-A that could be sequestered within the diverticulum. As expected, diverticulum volume increased with body mass (ln(diverticulum volume (μL)) = 2.45 (±0.51) ln(mass (g) of larva) +3.08 (±0.31), $F_{1,16}$ = 23, $P$ < 0.001, $r^2$ = 0.59).

No significant difference was found for mean frass (g) produced among the various sideroxylonal-A concentrations offered to sawflies ($F_{2,26}$ = 1.7, $P$ = 0.23), although frass production did vary among colonies ($F_{2,26}$ = 49, $P$ = 0.001) as well as among colonies over the months of the study ($F_{6,26}$ = 3.4, $P$ = 0.046) (Fig. 2*a–c*). A significant preference was found for leaves of the natal plant ($\chi^2$ = 8.2, $P$ = 0.004), independent of concentration of sideroxylonal-A in the natal plant (Fig. 3). Even when the concentration of sideroxylonal-A in the natal plant vastly exceeded the concentration of sideroxylonal-A of the leaves used for the feeding experiments, larvae produced significantly more frass while feeding on the natal plant, translating to a greater rate of feeding on host plant leaves than on the leaves of other plants offered.

### *Injections of sideroxylonal*

Recovery time following injection of sideroxylonal solution was significantly delayed only by the presence of methanol ($F_{3,54}$ = 16, $P$ = 0.000), as neither injection volume ($F_{1,54}$ = 0.01, $P$ = 0.8) nor the presence of sideroxylonal in the injection ($F_{3,54}$ = 0.4, $P$ = 0.8) had a significant effect on recovery time. The lack of response to injection volume suggested that no dose response was present. Slow recovery times were observed for larvae injected with methanol solutions.

### *Sideroxylonal concentrations in frass and regurgitant*

HPLC analyses of the concentration of sideroxylonal-A in frass and regurgitant samples showed very broad sideroxylonal peaks (Fig. 4*a–c*) (retention time ≈ 4 min). In some analyses, a complex of peaks was visible (Fig. 4*b*), indicating that compounds other than sideroxylonal-A are present. The sideroxylonal-A peaks for the frass samples were so low (Fig. 4*a*, *b*) as to suggest that there was minimal sideroxylonal-A present. The sideroxylonal peaks for the regurgitant samples were relatively high (Fig. 4*c*), indicating extremely high concentrations of sideroxylonal-A. The converted concentration (mean ± s.d.) for frass was 0.2 ± 0.02 mg sideroxylonal-A (g dry mass)$^{-1}$ ($n$ = 39) and for regurgitant was 48.3 ± 19.0 mg sideroxylonal-A μL$^{-1}$ ($n$ = 5).

### *Laboratory analysis for sideroxylonal-A budget*

Using the measures of rate of frass production, diverticulum volume and the appropriate concentrations of sideroxylonal-A in the leaves provided, a regression for sideroxylonal-A was estimated for the feeding studies between ingestion and both storage and faecal loss (Fig. 5). The regressions showed that a significant relationship existed between the quantity of sideroxylonal-A consumed (independent variable), the quantity of sideroxylonal-A present in the frass, and the amount in the fluid within the diverticulum (Fig. 5). Most consumed sideroxylonal-A was stored in the diverticulum (80–100%). Loss of sideroxylonal-A with frass did increase with increased leaf consumption, but overall a negligible

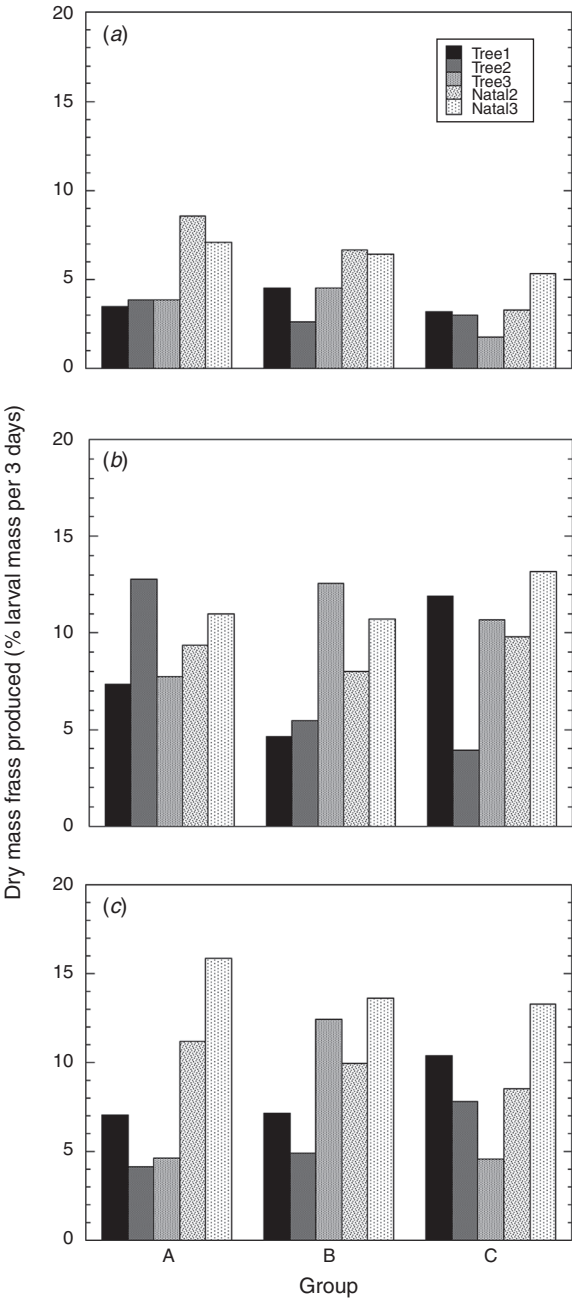


**Fig. 2.** Frass produced per cluster of larvae per three-day period, as a percentage of cluster mass. Tree1, Tree2 and Tree3 in the legend refer to leaves containing the various concentrations of sideroxylonal-A used for feeding studies. Natal 2 and 3 refer to periods during which larvae were fed on their host plants. The three groups used in each study are indicated by letters (A, B and C) with the three graphs representing feeding studies done in (*a*) April, (*b*) July and (*c*) August.

quantity of sideroxylonal-A was lost in the frass (<1%) relative to the amount consumed. A more detailed budget for the third feeding study (fifth-instar larvae) was developed using the amount of sideroxylonal ingested and either stored in diverticula or lost in faeces (Table 3). Clearly, most of the ingested sideroxylonal is located within the diverticula, with a

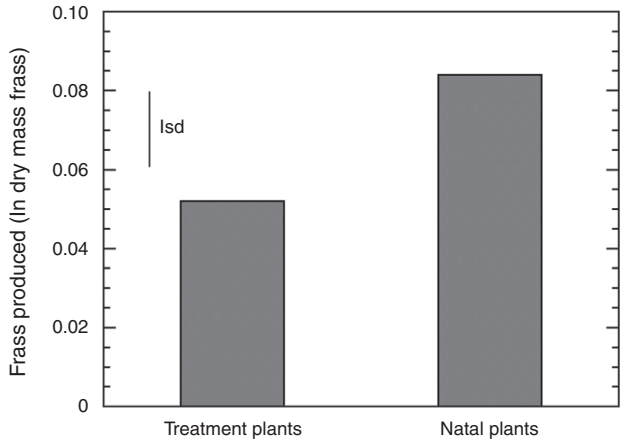


**Fig. 3.** Mean frass production (natural log-transformed) for animals feeding on experimental treatment and host plants (l.s.d., least significant difference).

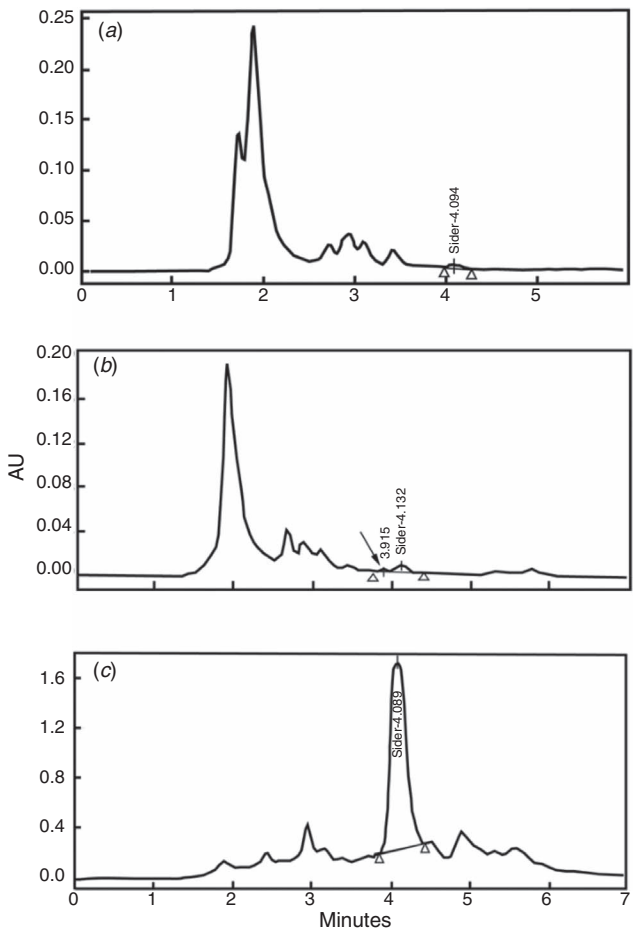


**Fig. 4.** High-performance liquid chromatograms for frass (*a* and *b*) and fluid (*c*) from the diverticulum of sawflies. Peaks that correspond to sideroxylonal-A are small in frass samples, but a large peak from diverticula fluid occurs at the sideroxylonal-A location. The arrow in (*b*) shows an additional peak suggesting that another compound that elutes near sideroxylonal-A is present.

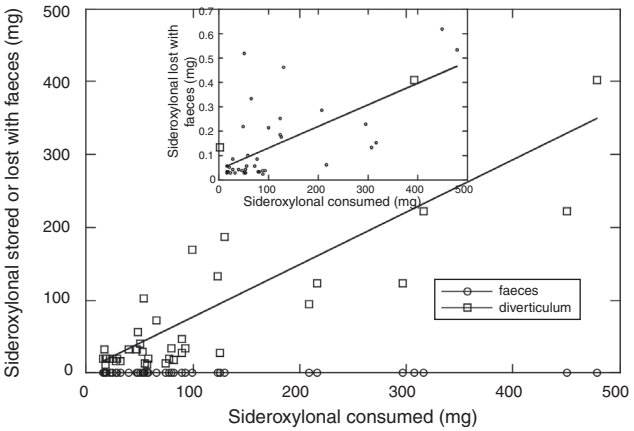


**Fig. 5.** Regression between total sideroxylonal consumed (mg per three days) and amount of sideroxylonal stored in the diverticulum and lost with frass (mg per three days). The regression fit for the frass is so close to the origin that it is obscured. Insert figure shows regression for frass with *y*-axis scaled appropriately to show relationship with sideroxylonal-A. Amount of sideroxylonal in diverticulum (mg) = 4.54 (±13.9) + 0.72 (±0.09) amount of sideroxylonal consumed (mg) ($F_{1,38} = 66.31, P < 0.001, r^2 = 0.64$). Amount of sideroxylonal lost in frass (mg) = 0.043 (±0.03) + 0.0009 (±0.0002) amount of sideroxylonal consumed (mg) ($F_{1,38} = 25.52, P < 0.001, r^2 = 0.41$). Values in parentheses are standard errors of the estimates of intercept and slope.

**Table 3. Estimated daily intake, storage and loss of sideroxylonal-A for fifth-instar sawfly larvae from the third feeding study**

Data for metabolism were determined by difference between intake and sum of storage and loss. Data presented are amount of sideroxylonal ingested over the whole period of the experiment per gram of sawfly (mean ± 1 s.e., $n = 15$)

| | Sideroxylonal-A (mg (g mass)$^{-1}$) | % intake |
|---|---|---|
| Intake | 16.5 ± 3.00 | 100 |
| Stored (diverticulum) | 13.8 ± 1.80 | 83.7 |
| Loss (faeces) | 0.06 ± 0.009 | 1.8 |
| Metabolised | 2.6 | 16 |

negligible amount lost in faeces. However, some breakdown of the sideroxylonal appears to occur as a result of metabolism.

## Discussion

Sideroxylonal-A in the leaves neither stimulated nor inhibited frass production of larvae of *P. affinis affinis*. Larval performance was best when they were offered leaves of the tree from which larvae were originally collected. A large proportion of ingested sideroxylonal-A is stored in the diverticulum, with a negligible quantity passing through the digestive system unmodified. The large size of the diverticula would permit storage of oils and other compounds that might interfere with digestion.

Frass production in larvae was relatively constant despite increased concentrations of sideroxylonal-A in the food, indicating that the rate of feeding was not affected by sideroxylonal-A concentration in leaves *per se*. A similar

response has been indicated to occur with paropsine beetle larvae (Marsh *et al*. 2017), and a last-instar caterpillar (*Hyalarcta huebneri*) tolerated high concentrations of sideroxylonal-A in the diet (Cooper 2001). In contrast, feeding in other species of insects may be affected by sideroxylonal concentrations in the foliage. High concentrations of sideroxylonal-A have been suggested to inhibit feeding in Christmas beetles (Matsuki *et al*. 2011*a*). Thus, sideroxylonal may deter feeding only in some insect species and only for specific instars.

The sideroxylonal budget indicated that a negligible proportion of ingested sideroxylonal is excreted unmodified by sawfly larvae. Morrow *et al*. (1976) used gas–liquid chromatography to measure the quantity of oils in leaf material, regurgitant, midgut material, and faecal material in larval *P. affinis affinis* and found that excretion accounted for a minimal proportion of ingested oil. The chromatograms for oils of midgut and faecal matter showed less complexity than chromatograms for leaf matter and oral fluid (Morrow *et al*. 1976), and our measurements of sideroxylonal-A are similar, suggesting a relatively small quantity of sideroxylonal in the faeces. In contrast, larvae of *H. huebneri* produced faecal pellets containing as much as 25% of the sideroxylonal-A they ingested (Cooper 2001). Larvae of *P. affinis affinis* are thus capable of metabolising sideroxylonal-A, or of sequestering and storing sideroxylonal-A before it reaches the gut, similar to the mechanisms for terpenes reported for diprionid and other pergid sawflies (Eisner *et al*. 1974; Schmidt *et al*. 2000, 2010; Schmidt and Walter 2011). However, even if the unmetabolised compound enters the hæmolymph, the lack of effect of the sideroxylonal following injections suggests that no toxic effect is present.

The ability to divert and store secondary metabolites before they enter the digestive system allows an insect to avoid the effects of a potentially large proportion of ingested PSMs without having to vary its feeding behaviour. The results of the HPLC analysis of sawfly regurgitant indicate that most ingested sideroxylonal-A is stored in the diverticulum. This fate is similar to that for plant essential oils in the diet, as reported for other pergid sawflies (Morrow *et al*. 1976; Schmidt *et al*. 2000). However, the capacity of the diverticulum in dissected individuals may be greater than what was measured assuming all the isotope was equilibrated with the volume held within the diverticula. Using a single lobe (Fig. 1*b*), we calculated that the volume was more than double what our regression suggested. Presumably, not all material within the diverticula can be regurgitated, and therefore may not be involved in defensive responses. Potentially, that would mean that little or no plant secondary compounds are metabolised in the digestive system, but all PSMs would be removed and stored in diverticula.

Is the absence of PSMs in the digestive system necessary to avoid toxic effects, such as occurs in beetle (*Paropsis atomaria*) larvae (Henery *et al*. 2008)? The injection of sideroxylonal-A directly into the hæmocoel had no effect on larvae, suggesting that even if absorption of these compounds before metabolism occurs, no toxic effects are present. However, the low solubility of sideroxylonal-A may limit any absorption within the digestive system. Our estimate of sideroxylonal excreted in frass or metabolised is 20%, and our micrographs do not suggest that any obvious plant oil glands are present in the digestive system. Possibly, any PSMs that are in the digestive system precipitate out of solution and therefore would not be absorbed through the peritrophic membrane that is present in the midgut (Fig. 1*c*). We did not observe any damage to the peritrophic membrane, as reported for *P. atomaria* feeding on resistant *Eucalyptus grandis* (Henery *et al*. 2008) regardless of sideroxylonal-A content.

The sawfly gut may have some capacity to metabolise sideroxylonal-A, as suggested by the small second peak in the frass (Fig. 4*b*), and this pattern may be similar to the detoxification of 1,8-cineole previously reported (Schmidt *et al*. 2000). Schmidt *et al*. (2000) found that the concentration of essential oils such as cineole decreased significantly between the foregut and the midgut in an undescribed *Pergagrapta* species, supporting previous suggestions that the midgut is the location of PSM detoxification in pergid sawflies (Rose 1987). Differential metabolism of 1,8-cineole was reported to occur in four different species of eucalypt-feeding beetles, as indicated by the variation in excreted cineole compounds (Southwell *et al*. 2003).

Host plant feeding regardless of concentration of sideroxylonal-A supports that this compound does not affect feeding rate in these larvae. Even in the third feeding study, when sideroxylonal concentration in the natal plant was higher than any experimental concentration, faecal production rate was higher on leaves from their natal plant. Although we did not perform a preference test, our results suggest that sawfly feeding is triggered by host plant recognition as opposed to deterrents. Occasionally, pergid sawfly larvae will defoliate a tree and move to a neighbouring tree (Carne 1962), which could change their feeding rate. Further work is needed to determine what happens in those situations and the influence that has on larval development.

Two species of pine sawfly (Diprionidae) have been reported to avoid young foliage with high concentrations of two resin acids by eating the old foliage of jack pine, *Pinus banksiana* (Ikeda *et al*. 1977), indicating that plant deterrents could affect feeding in that sawfly. The key difference between diprionid and pergid sawflies is that although the diverticulum of pergid larvae opens into the mouth (pharyngeal diverticulum), the diverticulum of the diprionid sawflies opens further back in the oesophagus (Maxwell 1955). PSMs may have greater opportunity to affect the diprionid digestive system than the pergid system, and so the diprionids may be more selective in food choices, although resin acids are stored in their diverticula as well.

However, what aspects of the host plant can stimulate the higher rate of feeding in larval sawflies? The various sensory mechanisms involved in feeding of pergid sawfly larvae and natal host plant recognition require further investigation. The large size of the diverticula also may indicate that this organ may be capable of doing more than just acting as a defensive and storage system. Future work is required to examine whether the diverticula may be capable of fermenting or metabolising compounds that are present, possibly as an energy source either to aid growth or moulting.

## Conflicts of interest

The authors declare no conflicts of interest.

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