Can insect egg deposition ‘warn’ a plant of future feeding damage by herbivorous larvae?

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Plant anti-herbivore defence is inducible by both insect feeding and egg deposition. However, little is known about the ability of insect eggs to induce defences directed not against the eggs themselves, but against larvae that subsequently hatch from the eggs. We studied how oviposition (OP) by the sawfly *Diprion pini* on *Pinus sylvestris* foliage affects the plant’s defensive potential against sawfly larvae. Larvae that initiated their development on *P. sylvestris* twigs on which they hatched from eggs gained less weight and suffered higher mortality than those fed on egg-free twigs. The poor performance of these larvae also affected the next herbivore generation since fecundity of resulting females was lower than that of females which spent their larval development on egg-free pine. Transcript levels of *P. sylvestris* sesquiterpene synthases (*PsTPS1, PsTPS2*) were increased by *D. pini* OP, reached their highest levels just before larval hatching, and decreased when larvae started to feed. However, concentrations of terpenoid and phenolic metabolites presumed to act as feeding deterrents or toxins for herbivores did not change significantly after OP and feeding. Nevertheless, our performance data suggest that insect egg deposition may act to ‘warn’ a plant of upcoming feeding damage by larvae.

**Keywords:** plant defence; herbivory; insect oviposition; performance; sesquiterpene synthases; terpenoids

1. INTRODUCTION

Herbivore damage induces various types of defences in plants (e.g. [1–8]). In direct defence, herbivore feeding can induce an increase in chemical defences or a decrease in plant nutritional quality and thus lead to reduced performance of herbivores (e.g. [9,10]). In indirect defence, herbivory can induce changes in emission of volatile compounds that attract antagonists of the herbivores (e.g. [11]). Besides responding to herbivore feeding, plants also react to the egg deposition of herbivorous insects by mounting direct and indirect defences against the eggs. Direct plant defences against eggs range from detachment of eggs by hypersensitive response of a leaf at the site of egg deposition to oviposition (OP)-induced production of an ovicidal substance killing the eggs (reviewed in [12–15]). Indirect plant defence against insect eggs involves the attraction of egg parasitoids by OP-induced plant volatiles (reviewed in [12–15]). So far, little attention has been paid to whether insect egg deposition can also trigger responses directed against herbivores that hatch from those eggs. In most studies on the impact of plant defence on insect performance, insect larvae are placed on an egg-free plant, whereas in nature newly hatched larvae may have to cope with plant quality that has been influenced by their eggs.

Here, we tested the hypothesis that plant defence induced by insect egg deposition affects the feeding larvae. We compared the herbivore performance on plants with and without eggs for an entire generation. In our plant–herbivore system, the plant is well known to be inducible by insect egg deposition. Defence of Scots pine, *Pinus sylvestris*, is induced by eggs of the herbivorous pine sawfly *Diprion pini*. Sawfly egg deposition on pine needles induces both locally and systemically the emission of pine terpenoid volatiles that attract egg parasitoids [16,17]. The egg-laying sawfly female slits a pine needle with her sclerotized ovipositor valves and inserts the eggs into the slit. Artificial wounding mimicking this slitting of the needle during OP does not induce the emission of volatiles attractive to the parasitoid [16]. While volatiles released 3 days after egg deposition are attractive to the parasitoid, volatiles released a day earlier or a day later are not attractive [18]. About 14 days after egg laying, larval hatch and feed gregariously on pine needles. While adults do not feed at all, feeding of larvae may cause severe damage in pine forests during mass outbreaks [19].

Previous work has investigated the *P. sylvestris* terpene synthase enzymes that are putatively involved in the formation of terpene volatiles [18,20]. The expression of the sesquiterpene (ST) synthase genes *PsTPS1*, encoding an (E)-β-caryophyllene/α-humulene synthase, and...
2. MATERIAL AND METHODS

(a) Plant and insect material

Plant material of *P. sylvestris* was collected in forests near Berlin, Germany. Branches were taken from mature trees (greater than 15 years old), cleaned according to a method of Moore & Clark [22] and kept in water at 10 °C prior to needle analysis or use in performance studies. Although detached branches may have different properties than intact ones, detached *P. sylvestris* twigs have been previously demonstrated to have defenses inducible by *D. pini* egg deposition and jasmonic acid; egg-induced twigs were shown to attract egg parasitoids [16–18,23]. We did not work with young, potted trees, because *D. pini* is not known to infest pine trees younger than 10 years [24]. *Diprion pini* was reared in the laboratory following the methods described by Bombosch & Ramakers [25].

(b) Performance studies

Sawflies were offered pine branches in a climate chamber (20 °C, 18 L: 6 D, approx. 37 μmol photons m−2 s−1, 70% r.h.). Branches from 10 trees were used, although not every performance parameter was tested on each twig (figure 1). A branch from each tree was divided into three twigs (about 45 cm long), which were kept in tap water. One twig was used for each of the three different treatments: A, B and C. Using plant material from the same tree for every treatment was a way to limit the variation in chemical properties known for every treatment was a way to limit the variation in chemical properties known for

<table>
<thead>
<tr>
<th>parameter</th>
<th>gender</th>
<th>median (interquartile ranges) #</th>
<th>unit</th>
<th>N (plants)</th>
<th>N (insects)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>larval weight</td>
<td>♀♂</td>
<td>6.3 (5.0–8.1)</td>
<td>7.3 (5.8–9.1)</td>
<td>10⁻¹ mg</td>
<td>7</td>
<td>676/424 &lt; 0.001</td>
</tr>
<tr>
<td>larval survival</td>
<td>♀</td>
<td>26</td>
<td>70%</td>
<td>3</td>
<td>417/247</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>larval development time</td>
<td>♀</td>
<td>29 (27–31)</td>
<td>28 (25–31) days</td>
<td>6</td>
<td>118/147</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>24 (22–27)</td>
<td>24 (21–28) days</td>
<td>6</td>
<td>140/119</td>
<td>n.s.</td>
</tr>
<tr>
<td>cocoon phase</td>
<td>♀</td>
<td>12 (9.5–13.0)</td>
<td>12 (9.8–12.0) days</td>
<td>6</td>
<td>56/63</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>12 (10.0–13.0)</td>
<td>12 (11.0–13.0) days</td>
<td>6</td>
<td>102/67</td>
<td>n.s.</td>
</tr>
<tr>
<td>cocoon weight</td>
<td>♀</td>
<td>130.4 (117.0–143.2)</td>
<td>141.9 (128.9–153.4) mg</td>
<td>6</td>
<td>118/147</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>65.2 (55.2–72.4)</td>
<td>68.5 (61.8–76.5) mg</td>
<td>6</td>
<td>140/119</td>
<td>= 0.001</td>
</tr>
<tr>
<td>adult weight</td>
<td>♀</td>
<td>70.5 (60.0–83.4)</td>
<td>81.0 (74.0–87.4) mg</td>
<td>6</td>
<td>56/63</td>
<td>= 0.002</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>24.3 (20.3–27.1)</td>
<td>28.3 (21.9–28.3) mg</td>
<td>6</td>
<td>102/67</td>
<td>n.s.</td>
</tr>
<tr>
<td>female fecundity</td>
<td>♀</td>
<td>100 (92.0–115.0)</td>
<td>114 (103.5–126.5) eggs</td>
<td>3</td>
<td>27/35</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Figure 1. Performance of *D. pini* after larval development on *P. sylvestris* twigs which had either suffered oviposition (OP larvae, left/black) or had non-oviposition (NOP larvae, right/grey). Medians were compared using the Mann–Whitney U-test. Larval survival data (onset L1 to pupation) were analysed by a χ²-test. Not significant (n.s.; *p* > 0.05). Of the two values given for *N* (insects), those on the left are for OP and those on the right for NOP individuals. Hash (#) denotes: medians and interquartile ranges are shown for all values except for larval survival given in % (*N survival data = 100%*).

PTPS2, encoding a 1(10),5-germacradien-4-ol synthase, was induced by eggs of *D. pini*. Increase in transcript levels of these genes coincided with the attractiveness of egg-laden foliage. *P. sylvestris* has been shown to produce jasmonic acid, which is known to be involved in the defense against herbivory. In addition, water content and foliage C/N ratio were measured, which provides some general information on the nutritive value of the needle material ([21] and references therein).
fewer than four egg clusters were laid. As soon as larvae hatched on twig C, they were gently transferred to twig A using a blunt, sterilized needle. Larvae on twig A then began to feed on twigs that had not experienced any OP previously. We refer to these larvae as ‘non-oviposition’ (NOP) larvae. The larvae hatching on twig B were not transferred to another twig, but instead were moved after hatching to a part of the same twig a few centimetres away from the hatching site. These larvae now fed on twigs that had experienced OP, and we refer to them as ‘OP larvae’. OP larvae were transferred within twigs to control for manipulations experienced by NOP larvae.

When NOP and OP larvae had consumed all the foliage on their initial twigs (after about two weeks), they were transferred to fresh twigs of P. sylvestris. These new twigs were the same for both groups of larvae and had experienced no prior OP. This transfer to fresh twigs reflects the situation in nature where, in later larval stages, D. pini larvae move away from the twig where they fed during their first larval stages. Thus, OP-induced effects on plant quality are experienced directly only by young larvae. The entire larval development takes about four weeks at the conditions used here. The performance parameters measured were larval weight (2 days after hatching), time of larval development (from hatching to onset of pupation, i.e. spinning a cocoon), larval survival (i.e. survival from L1 to onset of pupation); cocoon weight, length of cocoon phase and adult weight immediately after emergence. Adult sawflies do not feed. Females (1 day-old) were dissected, and the number of eggs in their ovaries was also counted.

(c) Treatment of pine twigs for molecular and chemical analysis

Five P. sylvestris twigs were cut from one branch and subjected to five different treatments as follows: (i) untreated control (C): the twig remained uninfested until harvesting of needles; (ii) egg deposition 3 days (E3): the twig received eggs by D. pini 3 days before harvesting of needles. In order to obtain egg-laden pine twigs, we placed three males and females of D. pini on the twig for about 24 h. Females laid their eggs on the needles within this time period (compare; performance studies); (iii) egg deposition 14 days (E14): the twig received eggs 14 days before harvesting of needles as described for (E3); (iv) egg deposition plus feeding (E + F): the twig received eggs, but needles were not harvested until 2 days after larval hatching. Hence, the twig was induced by both egg deposition and larval feeding; (v) larval feeding (L): the twig did not receive any eggs, but larvae that hatched on other twigs were transferred to the twig 2 days before harvesting of needles.

In total, for each treatment, seven different branches were taken from seven different trees (seven biological replicates). After treatment, pine needles were removed from the twigs, transferred to liquid nitrogen and kept at −80°C until analysis. General conditions of plant storage before and during treatments were the same as described above.

(d) Molecular analysis

Harvested needles of the differently treated pine twigs were ground, and RNA was extracted with the Invisorb Spin RNA Mini Kit (Invitek, Berlin, Germany). Synthesis of cDNA and measurements of transcript levels of PsTPS1 and PsTPS2 by quantitative real-time PCR followed the methods described by Köpke et al. [18]. Each sample (five treatments × seven biological replicates) was measured at least three times (three technical replicates).

(e) Chemical analysis

(i) General

We used for the chemical analyses needles from the same P. sylvestris twigs that had been subjected to the five treatments described above and used for the molecular analysis.

(ii) Mono- and sesquiterpene extraction

Terpenes were extracted from pine needles (200 mg) as described by Martin et al. [27]. Sample preparation was continued as described by Köpke et al. [18] except for the internal standard, which was methylcaprylate (150 µg ml⁻¹) in the present study. The column was washed with 1 ml of tert-butyl methyl ether, and the combined eluate was collected in a clean vial, evaporated under gaseous nitrogen to an approximate volume of 250 µl and then stored at −20°C until analysed by gas chromatography–mass spectrometry (GC–MS).

(iii) Diterpene extraction

Frozen and ground needle material (100 mg) was extracted in 1.5 ml tert-butyl methyl ether, including the internal standard tetrahydroabietic acid (34 µg ml⁻¹), by shaking the samples constantly for 14 h. The extraction was continued as described above for monoterpenes (MT) and ST extraction. However, here the eluate (300 ml) was methylated by adding 37.6 µl trimethylsulphonium hydroxide. Derivatized samples were subjected to GC–MS analysis.

(iv) Phenolic extraction

Pine needles were ground to a fine powder under liquid nitrogen and lyophilized. A sample of approximately 80 mg of the powder was extracted for 12 h at 4°C with 4 ml methanol containing 10 µg ml⁻¹ chlorogenic acid as internal standard. The extract was filtered, dried under nitrogen and re-dissolved in 1 ml methanol. A 1:10 dilution in methanol was analysed using liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS). Here, we analysed three additional biological replicates for each treatment plus the seven biological replicates we used in all other analyses (i.e. 10 branches taken from 10 different trees).

(v) GC–MS analyses of terpenoids

Ether-needle extracts (1 µl) were analysed on a GC–MS system with a HP–5 capillary column as listed in Köpke et al. [18].

—Mono- and sesquiterpenes. The GC–MS was set at an injector temperature of 220°C. The temperature programme started with 40°C for 2 min, raised to 210°C (5°C min⁻¹), and raised further to 300°C (60°C min⁻¹ 2 min hold; helium flow: 2 ml min⁻¹). The MS detector was operated using the total ion mode at a temperature of 230°C. The products were identified as described in Köpke et al. [18].

—Diterpenes. The GC–MS was set at an injector temperature of 270°C. The temperature programme started with 150°C for 3 min; afterwards it rose to 280°C (3.5°C min⁻¹) and was held for 4 min. The MS detector was operated using the total ion mode at a temperature of 230°C. The products were identified by comparing mass spectra and retention times with those in the literature and in the Wiley 275-L or NIST 98.1 MS libraries. The identity of diterpenes (DT) was further verified by comparison with commercially available authentic standards.
MT, ST and DT were quantified in pine needle extracts relative to the corresponding internal standard. The relative quantities of these compounds were then averaged from the seven independent samples of each treatment. For each sample, three technical replicates were analysed in the case of MT and ST and two replicates in the case of DT.

(vi) LC–MS/MS analyses of phenolic compounds

Chromatography was performed on an Agilent 1200 high performance liquid chromatography (HPLC) system (Agilent Technologies). Separation was achieved on a Kinetex C18 column with 100 x 4.6 mm dimensions and a particle size of 2.6 μm (Phenomenex, Aschaffenburg, Germany). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0–1 min, 100% A; 1–7 min, 0–65% B in A; 7–8 min 65–100% B in A; 8–9 min 100% B and 9–10 min 100% A. The total mobile phase flow rate was 1.8 ml min⁻¹. The column temperature was maintained at 25°C.

An API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbospray ion source was operated in the negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards, where available. For dimeric proanthocyanidins, partially purified plant extracts were used for optimization. The ion spray voltage was maintained at −5500 eV. The turbo gas temperature was set at 700°C. Nebulising gas was set at 70 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 10 psi. Scheduled multiple reaction monitoring (MRM) was used to monitor the conversion of the parent ion → product ion for each quantified analyte: m/z 302.8 → 179 (collision energy (CE) − 28 V, declustering potential (DP) [28] − 55 V) for quercetin; m/z 308.2 → 125.1 (CE − 28 V, DP − 40 V) for taxifolin; m/z 288.9 → 109.1 (CE − 34 V, DP − 30 V) for catechin; m/z 304.8 → 179 (CE − 28 V, DP − 390 V) for gallicatechin; m/z 341.9 → 107.1 (CE − 46 V, DP − 60 V) for isorhamnetin; m/z 352.8 → 191.1 (CE − 24 V, DP − 25 V) for chlorogenic acid; m/z 430.8 → 268 (CE − 46 V, DP − 80 V) for apigenin glucoside; m/z 462.9 → 300 (CE − 40 V, DP − 390 V) for quercetin glucoside and quercitin galactoside; m/z 464.8 → 125.1 (CE − 44 V, DP − 395 V) for taxifolin glucoside; m/z 576.9 → 289.1 (CE − 30 V, DP − 50 V) for proanthocyanidin B1; m/z 592.9 → 125.1 (CE − 52 V, DP − 400 V) for the catechin : gallocatechin dimer; m/z 609 → 125.1 (CE − 50 V, DP − 45 V) for the gallocatechin dimer. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionisation efficiencies was verified by analysing a dilution series of pine needle extracts. External calibration curves for catechin, taxifolin and apigenin glucoside were created by linear regression. Flavan-3-ol concentrations were determined relative to the catechin calibration curve, flavonoids relative to taxifolin, and flavanoid glycosides relative to apigenin glucoside.

(vii) C/N ratio measurement

Every pine needle sample (4–5 g) was ground in a mortar, lyophilized and later pulverized with a Retsch mill (MM200, Retsch, Düsseldorf, Germany) for 3 min (frequency: 30). The pulverized samples were poured in paper bags and kept over night in a 60°C chamber for further dehydration. The next day the samples were kept for 1 h in a desiccator to adjust to room temperature. Then each sample (18–19 mg) was poured into a zinc bowl, and the C/N ratio was determined using a ‘Vario EL II’ (Elementar Analyensysteme GmbH, Hanau, Germany) at the Max Planck Institute for Biogeochemistry, Jena, Germany.

(viii) Water content

Needle samples of 100 or 200 mg fresh weight were dried in an 80°C oven for ca 2 days. The needle water content was calculated by subtraction of dry weight from initial fresh weight.

(f) Statistical analysis

Performance data of NOP and OP larvae were statistically compared by using the Mann–Whitney U-test, except for larval mortality data that were evaluated by a χ²-test. Comparison of molecular and chemical data obtained from the five differentially treated twigs taken from the same branch was done by a Friedman ANOVA; the Wilcoxon–Wilcoxon test was used for post hoc comparisons. All statistical tests were performed using the statistical software StatSoft, v. 1999, Statistica for Windows (Tulsa, OK, USA; see the electronic supplementary material, table S1).

3. RESULTS

(a) Performance studies

Comparison of the performance of sawfly larvae that started their larval development on twigs that had experienced OP to the performance of larvae that fed on twigs that had suffered no prior OP (NOP) showed striking differences. OP larvae performed worse than the NOP larvae (figure 1). Two day-old OP larvae gained less weight than NOP larvae (about 0.6 mg compared with 0.7 mg), and significantly fewer OP larvae (26%) survived larval development than NOP larvae (70%). Thus, larval survival from L1 to the final larval instar was reduced by over 60 per cent when larvae started their development on egg-laden pine twigs compared with egg-free twigs. The duration of larval development and cocoon phase of OP and NOP D. pini did not differ. However, the weight of OP cocoons was lower than that of NOP cocoons. The weight of female OP cocoons was on average more than 8 per cent lower than that of female NOP cocoons. For males, the weight of OP cocoons was on average about 6 per cent lower than that of NOP cocoons. After emergence, adult OP females were on average more than 12 per cent lighter than NOP females. OP females produced fewer eggs than NOP females (about 100 compared with 114).

(b) Molecular analysis

Transcription of the P. sylvestris ST synthases, PYP1S1 and PYP2S2, encoding an (E)-β-caryophyllene/α-humulene synthase and a 1(10),5-germacradien-4-ol synthase, respectively, was significantly enhanced in twig samples of all OP and larval feeding treatments when compared with the untreated control (C) (figure 2). Increased transcription of both of these genes 3 days after egg deposition (E3) had been shown previously by Köpke et al. [18]. At 14 days after OP (E14), shortly before hatching, the transcript levels of both genes were several-fold higher than 3 days after OP. However, as soon as larvae hatched from eggs and had fed for 2 days on a twig with prior eggs (E + L), transcript levels declined. This was significant for PYP1S1, but for PYP2S2 because of the high variability of transcript levels.
Table 1. Concentrations of monoterpenes (MT), sesquiterpenes (ST), diterpenes (DT) and phenolic compounds (PCs) in needles of *P. sylvestris* after different *D. pini* OP and feeding treatments. Treatments: C, control, no treatment; E3, twigs laden with eggs of *D. pini* harvested 3 days after OP; E14, twigs laden with eggs harvested 14 days after OP, shortly before hatching; E + L, twigs on which larvae hatched from eggs and fed for 2 days; L, twigs that never carried eggs, but were fed on by larvae for 2 days.

<table>
<thead>
<tr>
<th>Parameter measureda</th>
<th>C</th>
<th>E3</th>
<th>E14</th>
<th>E + L</th>
<th>L</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Terpenoids</strong> (mg g−1 DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total MT and ST</td>
<td>17.20 (3.34)</td>
<td>16.88 (2.73)</td>
<td>17.96 (2.72)</td>
<td>17.92 (2.90)</td>
<td>17.70 (2.60)</td>
<td>n.s.</td>
</tr>
<tr>
<td>total DT</td>
<td>0.0114 (0.0020)</td>
<td>0.0128 (0.0017)</td>
<td>0.0139 (0.0019)</td>
<td>0.0124 (0.0017)</td>
<td>0.0112 (0.0012)</td>
<td>n.s.</td>
</tr>
<tr>
<td>total PC (mg g−1 DW)</td>
<td>0.776 (0.16)</td>
<td>0.740 (0.14)</td>
<td>0.760 (0.13)</td>
<td>0.667 (0.11)</td>
<td>0.845 (0.15)</td>
<td>n.s.</td>
</tr>
<tr>
<td>water content</td>
<td>56.88 (0.94)</td>
<td>62.42 (0.62)</td>
<td>63.77 (0.613)</td>
<td>62.09 (0.81)</td>
<td>59.51 (1.217)</td>
<td>n.s.</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>33.58 (1.51)</td>
<td>33.87 (1.76)</td>
<td>33.16 (0.88)</td>
<td>35.19 (0.75)</td>
<td>34.24 (1.78)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

aValues are given as mean (s.e.) of three technical replicates of each of at least seven biological samples, except for total PC and C/N measurements. Only one technical replicate of seven biological replicates was analysed in case of C/N measurements. For phenolic content analyses, one technical replicate of 10 biological replicates was analysed. Not significant (n.s.; p > 0.05), Friedman ANOVA. DW, dry weight; FW, fresh weight.

Twigs with prior eggs and feeding damage showed lower transcript levels of *P. TPS1* than twigs with feeding damage, but no egg deposition.

**4. DISCUSSION**

In our study of pine (*P. sylvestris*)–sawfly (*D. pini*) interactions, we found strong evidence that egg deposition might be a cue for mounting plant defences against larvae expected to hatch from the eggs. The performance of sawfly larvae on pine twigs where they hatched from eggs was much poorer than on twigs that did not have prior eggs, and the resulting females produced significantly fewer eggs. Hence, the detrimental effects on larvae feeding on pine with prior eggs carry over into the next generation —see reduced weight of 2 day-old OP larvae. The performance disadvantages suffered during early life might be a cue for mounting plant defences against larvae expected to hatch from the eggs. The young larvae of herbivores may be especially sensitive to detrimental environmental factors [28], and older larvae might not be able to overcome the performance disadvantages suffered during early life (see reduced weight of 2 day-old OP larvae).

In comparison with our results, Bruessow *et al.* [29] found that larval performance (measured by weight gain after an 8 day feeding period) of a specialist herbivore, the white cabbage butterfly *Pieris brassicae*, was not affected by prior treatment of an *Arabidopsis thaliana*
Sawfly larval feeding on pine with or without prior eggs induced expression of the two pine ST synthase genes when compared with untreated (egg-free) control pine (figure 2). This result is consistent with other studies showing that transcript levels of genes coding for terpene synthases are higher in tissue damaged by herbivore feeding than in undamaged controls [30–33]. Hence, the direction of feeding-induced transcriptional changes (increase and decrease) of these ST synthases depends on whether transcript levels of feeding-damaged tissue are compared with untreated control tissue or with the natural situation, i.e. egg-laden pine tissue (figure 3).

To get a more realistic picture of changes in the expression of terpene synthases and other defence genes in response to insect herbivory, we suggest that future studies use larvae that have hatched from eggs on the experimental plant, rather than just been placed on an egg-free plant, when this reflects the natural life history of the insect species under study.

The nutritional quality of egg-laden pine foliage was expected to be worse compared with egg-free foliage because of the decreased performance of sawfly larvae on pine with prior eggs. However, no significant differences were detected between P. sylvestris foliage with and without eggs in a variety of chemical parameters except for the PC taxifolin (see the electronic supplementary material). The lack of differences in the chemical composition of P. sylvestris after various OP and feeding treatments contrasts with the significant differences in sawfly performance observed in this study. The poor food quality of foliage after OP may be a result of changes in plant defence compounds or nutrients that we did not measure. Alternatively, perhaps terpene and phenolic contents changed significantly, but we did not sample at the right time points to detect these trends.

In conclusion, our results of the performance studies suggest that deposition of D. pini eggs on P. sylvestris acts as a warning for the plant leading to changes in foliage composition that make it a much poorer substrate for larval growth. Egg deposition was also shown to significantly increase transcription of terpene synthases just prior to larval hatching. Since no significant changes in phenolic chemistry, water content or C/N ratio were observed either, it might be premature to ascribe the negative effect of egg laying on larval performance to a defensive plant response. Nevertheless, as long as OP triggers declines in subsequent larval growth, weight and survival and adult fecundity, it can certainly be said to get the plant ‘ready for battle’ against feeding herbivores [34,35].

If insect eggs serve as a warning signal for future larval damage, this adds further to the multitude and complexity of plant responses to herbivory [1,36]. Given the costs and benefits of induced defences, plants are expected to adjust their defensive responses according to the risk of herbivory based on whatever reliable information they can perceive about the likelihood of future attack.
(e.g. [37]). Incidence of past herbivory upon a plant and volatiles released from feeding-damaged neighbouring plants have been shown to be used as cues that prime induced defences [34,35,38,39]. Our results suggest that deposition of insect eggs on a plant is also perceived as a diagnostic and very reliable feature of upcoming larval herbivory. Future studies are needed to clarify how defences induced by eggs interact with those induced by feeding larvae. Moreover, the ecological consequences of these interactive effects both for the plant and the herbivorous insect need further studies.

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