Plants can respond to insect infestation with defenses targeting insect eggs on their leaves and the feeding insects. Upon perceiving cues indicating imminent herbivory, such as damage-induced leaf odors emitted by neighboring plants, they are able to prime their defenses against feeding insects. Yet it remains unknown whether plants can amplify their defenses against insect eggs by responding to cues indicating imminent egg deposition. Here, we tested the hypothesis that a plant strengthens its defenses against insect eggs by responding to insect sex pheromones. Our study shows that preexposure of *Pinus sylvestris* to pine sawfly sex pheromones reduces the survival rate of subsequently laid sawfly eggs. Exposure to pheromones does not significantly affect the pine needle water content, but results in increased needle hydrogen peroxide concentrations and increased expression of defense-related pine genes such as SOD (superoxide dismutase), LOX (lipooxygenase), PAL (phenylalanine ammonia lyase), and PR-1 (pathogenesis related protein 1) after egg deposition. These results support our hypothesis that plant responses to sex pheromones emitted by an herbivorous insect can boost plant defensive responses to insect egg deposition, thus highlighting the ability of a plant to mobilize its defenses very early against an initial phase of insect attack, the egg deposition.

**Significance**

Plant defenses against herbivorous insects can target the feeding stages and the egg stage. Feeding-induced plant defenses are known to be primed by cues indicating imminent infestation, including sex pheromones. However, priming of egg-induced plant defenses has been unknown so far. Therefore, we studied whether a plant’s response to insect sex pheromones, which might indicate imminent egg depositions, primes defenses against the eggs. Indeed, exposure of pine to the sex pheromones of an herbivorous sawfly primes the tree’s defense against sawfly eggs. The priming effect results in enhanced egg mortality, enhanced accumulation of hydrogen peroxide in egg-laden needles, and differential expression of several defense-related pine genes. These findings open up exciting research perspectives in plant protection from insect infestation.

**Author contributions:** N.B., A.A.-C., and M.H. designed research; N.B., J.H., and A.A.-C. performed research; O.A. contributed new reagents/analytic tools; N.B., J.H., A.A.-C., and M.H. analyzed data; and N.B., J.H., A.A.-C., O.A., and M.H. wrote the paper.

The authors declare no competing interest.

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Data deposition: Sequences of *Pinus sylvestris* PCR products as well as the template accession numbers in Genbank for the primer design and the annotation information referred to in this paper have been deposited at the repository of the Max-Planck-Institute for Molecular Plant Physiology with open access at https://primedb.mpimp-golm.mpg.de/index.html?tid=reviewer&amp;pid=41fbcf5ceaa03476a56b57e4aeb5b8e261.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1910991116/#DCSupplemental.


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Our results show that exposure of pine to the sex pheromones of a female sawfly primes the tree’s defenses against sawfly eggs and results in enhanced egg mortality, enhanced accumulation of hydrogen peroxide in pine needles, and differential regulation of defense-related pine genes. These results provide evidence that plants are capable of strengthening their defense against a very early step of insect infestation, the egg deposition, by responding to cues preceding egg depositions.

Results and Discussion

Survival Rates of Sawfly Eggs Are Lower on Pine Previously Exposed to Sawfly Sex Pheromones. We compared survival rates of D. pini eggs that have been deposited on small, 3-year-old P. sylvestris trees previously exposed for 1 to 4 d to D. pini sex pheromones or, as a control, to the pheromone solvent hexane. After 24 h of pheromone (or hexane) exposure, D. pini females were allowed to oviposit for 1 d on the needles of these trees. A D. pini female inserts her eggs in a row (about 15 eggs per row) into a pine needle. After 12 to 14 d (egg incubation time), the larvae hatch from surviving eggs. We exposed the trees to a pheromone dose comparable to that which pine trees are exposed to during a mass D. pini outbreak (SI Appendix, Table S1). Exposure of pine trees to the pheromones significantly affected the pines’ resistance against sawfly eggs. The mean (±SE) survival rate of eggs on trees previously exposed to the pheromone (40.07 ± 2.89%) was significantly lower than the survival rate of eggs on untreated controls (60.37 ± 10.25%) and on trees exposed to the solvent hexane (59.65 ± 4.35%) (Fig. 1 and SI Appendix, Tables S2 and S3). The hexane treatment had no impact on the egg survival rate. This may be due to the high volatility of this solvent. Prior to treatment, the dispensers with hexane and the dispensers with pheromone dissolved in hexane were kept for 30 min in a fume cupboard, where the solvent evaporated; thereafter, pine trees were exposed to the dispensers for 24 h. The low survival rate of D. pini eggs on untreated trees in the absence of natural enemies and at favorable abiotic conditions indicates that P. sylvestris can directly defend itself against the eggs, as also suggested by an earlier study (18). The results here show that preexposure of pine to D. pini sex pheromones results in further reduction of the sawfly’s egg survival rate.

Pheromone Exposure Promotes Hydrogen Peroxide Accumulation in Egg-Laden Pine Needles. That preexposure of pines to sawfly sex pheromones significantly reduced survival of D. pini eggs raised the question of what causes this ecological effect. At the immediate interface between insect egg and plant, environmental humidity and leaf hydrogen peroxide concentrations are known to affect development of insect eggs and their survival (18, 20, 21). The humidity to which an insect egg is exposed is not only determined by air humidity but also by leaf water content. An increase in leaf hydrogen peroxide concentration and accumulation of other ROS in response to insect eggs may result in formation of necrotic plant tissue (22). This plant response provides an environment in which eggs of several insect species have been shown to suffer increased mortality (20, 23). Formation of necrotic tissue has been described for pines in response to D. pini egg deposition (18), but whether ROS accumulation in response to D. pini eggs is amplified by prior exposure of pines to pheromones is unknown.

Therefore, we investigated whether exposure of pine trees to pheromones 1) reduces the pine needle water content, thus possibly resulting in desiccation of the eggs, or 2) enhances the concentration of pine needle hydrogen peroxide concentrations, thus directly harming the eggs or resulting in amplified plant defense signaling (24). The needle water and hydrogen peroxide contents were analyzed 2 days after pheromone exposure, that is, 1 and 11 d, respectively, after egg deposition (Fig. 2 and SI Appendix, Fig. S1).

The water content of pine needles exposed to the different treatments was similar, and no significant differences between treatments were detected at any of the 2 measurement points after pheromone exposure (Fig. 24 and SI Appendix, Fig. S1A and Table S2).

Hydrogen peroxide accumulated in egg-laden needles at the end of egg incubation time (i.e., 11 d after egg deposition) (Fig. 2B). This egg-induced accumulation of hydrogen peroxide was significantly enhanced by the pheromone treatment 12 d earlier. In contrast, the pheromone treatment had no effect on the needle hydrogen peroxide concentration of the egg-free pines. Nor did exposure of the pines to hexane affect the needle hydrogen peroxide concentration (Fig. 2B and SI Appendix, Table S2). No induction of hydrogen peroxide accumulation was detectable shortly (1 d) after egg deposition. Nor did a preceding pheromone exposure affect the hydrogen peroxide concentration of pine needles shortly after egg deposition (SI Appendix, Fig. S1B and Table S2).

Thus, the pheromone-mediated strengthening of pine resistance against sawfly eggs is associated with enhanced accumulation of hydrogen peroxide in the pine needles, which becomes evident at the end of the egg incubation time. The enhanced hydrogen peroxide concentration might directly exert a detrimental effect on the eggs (20) and/or serve as an intensified early defense signal (24, 25). Several studies have shown an increase in plant hydrogen peroxide concentrations in response to wounding or herbivory (26–30) and to insect egg deposition (18, 20, 22). While a wound-induced increase in hydrogen peroxide concentration is known to be detectable almost immediately in response to herbivory (e.g., refs. 27 and 28), egg-induced increases have been observed only several days (22) after the egg treatment or at the end of the egg incubation time (18, 20). Here we show that exposure of a plant to a female insect sex pheromone (Fig. 2C), that is, an environmental cue indicating impending insect egg deposition, can even further promote the (egg) infestation-induced hydrogen peroxide accumulation.

Fig. 1. Impact of exposure of P. sylvestris to sex pheromones of pine sawflies (D. pini) on sawfly egg survival rates. (A) Percentage (mean ± SE) survival of D. pini eggs on untreated pine trees (n = 6), pine trees exposed to hexane (n = 8), and pine trees exposed to the pheromones (dissolved in hexane) (n = 8) for 24 h prior to egg deposition by 2 females per tree. Total number of eggs on untreated trees is 915 (mean number of eggs per tree ± SE: 152.5 ± 20.81), on hexane-treated trees is 1170 (mean ± SE: 148.6 ± 11.48), and on pheromone-treated trees is 858 (mean ± SE: 107.3 ± 11.76). Difference between numbers of laid eggs per the differently treated trees is not significant (n.s.) (ANOVA). Difference between numbers of laid eggs and hatched eggs within a treatment: ***P < 0.01; ****P < 0.001 (paired t tests). Different letters in bars indicate significant differences (P < 0.05) in survival rates among treatments (ANOVA followed by multiple pairwise t tests and Benjamini–Hochberg P value correction) (compare SI Appendix, Tables S2 and S3). (B) D. pini female on P. sylvestris. (C) Egg row of D. pini on a pine needle.
Pheromone Exposure Results in Changes of Expression of Defense-Related Pine Genes. To figure out whether, and if so how, exposure of pines to sawfly sex pheromones affects expression of defense-related pine genes, we ran qPCR analyses of needles from trees treated in different ways (Table 1 and SI Appendix, Table S4). Samples were harvested 2 and 12 d after pheromone or hexane exposure to differentiate between early and late treatment effects. We selected the following genes (for information on sequences, see SI Appendix, Tables S5 and S6): PsRboh (sequence homolog to a respiratory burst oxidase—plant NADPH oxidase), involved in ROS production; PsSOD (superoxide dismutase) encoding an enzyme catalyzing hydrogen peroxide formation; and PsCAT (sequence homolog to catalase) and PsAPX (sequence homolog to ascorbate peroxidase), both of which are involved in ROS degradation (25). We tested expression levels of a putative lipoygenase encoding gene (PsLOX) initiating the jasmonic acid (JA) pathway (31) and of PsPDF putatively encoding a plant defensin, which is inducible by early JA- and ethylene-mediated defense signaling (32). Additionally, we determined transcript levels of PsPR-1 (sequence homolog to pathogenesis-related protein 1), which is inducible by insect egg depositions on Arabidopsis thaliana (22, 33). Because accumulation of phenylpropanoid derivatives is involved in egg-mediated strengthening of antiherbivore defenses in several plant species (33–35), we also determined transcript levels of PsPAL encoding a putative phenylalanine ammonia lyase, an enzyme at the entrance of the phenylpropanoid pathway (36). As the hexane treatment did not affect expression of the genes tested (SI Appendix, Table S7), we normalized the gene expression levels of all other treatments to those determined for hexane-treated pines.

The pheromone exposure per se affected expression of only 2 of the 8 genes investigated. Shortly (2 d) after sawfly pheromone exposure, expression levels of PsRboh were significantly higher. Priming plants for improved resistance against phytopathogens by preexposure to pathogens or to priming chemicals such as β-aminobutyric acid also results in enhanced expression of RbohD in A. thaliana (37). In contrast to PsRboh, none of the other genes showed significantly altered transcript levels at this early time point after treatment. Twelve days after pheromone exposure, expression levels of PsPR-1 were significantly reduced in pheromone-treated trees.

Sawfly egg deposition without prior exposure of the pines to pheromones affected expression of pine catalase PsCAT, whose transcript levels were significantly higher only shortly after egg deposition, but not later. Expression levels of PsRboh coding for a putative ROS-generating enzyme and of PsAPX coding for a putative hydrogen peroxide detoxifying enzyme were lower at the later sampling time. When trying to relate these data to the hydrogen peroxide concentrations shown in Fig. 2B, these findings suggest that the high hydrogen peroxide levels in needles of egg-deposited pine trees are not due to Rboh-mediated production of ROS. This interpretation is in line with a previous study (22), which found no indication that Rboh is involved in hydrogen peroxide accumulation induced by application of butterfly egg extracts on A. thaliana. However, reduced degradation of ROS because of reduced availability of the ROS-degrading enzyme PsAPX at the end of the egg incubation period (Table 1) might at least contribute to the high hydrogen peroxide concentrations in egg-laden pine needles.

Interestingly, pheromone treatment followed by egg deposition resulted in enhanced expression of PsSOD-encoding superoxide dismutase, which catalyzes the formation of hydrogen peroxide. This result is in line with the higher hydrogen peroxide concentrations in pheromone-treated, egg-deposited needles at the end of the egg incubation period (i.e., 12 d after pheromone treatment). In contrast, expression levels of PsRboh, producing superoxide radicals as substrate for SOD, were low at the same time point in pheromone-treated, egg-deposited pine needles. Regulation of hydrogen peroxide concentrations may not only be mediated by the expression of genes encoding ROS generating and degrading enzymes. Also, the activation of these enzymes and other factors like a change in the abundance of ROS scavenging secondary compounds might have contributed to hydrogen peroxide accumulation in pheromone-exposed, egg-deposited needles. In A. thaliana, ROS accumulation is important for egg-induced up-regulation of PR-1 (22). In pines, expression of PsPR-1 was significantly up-regulated in the pheromone-exposed, egg-deposited needles with the highest hydrogen peroxide concentrations. These results suggest that
Expression of finding supports the growing evidence that these hormones can enhance in trees preexposed to pheromones and subsequently to sawfly egg deposition. Hence, the pheromone exposure resulted in a significant up-regulation of both a salicylic acid (SA)-responsive and a jasmonic acid (JA)-responsive gene, among those important in processes such as the formation of both JA- and SA-mediated priming of plant defense against insect egg deposition. Despite numerous studies showing antagonistic interactions between JA- and SA-mediated plant defenses (38), our finding supports the growing evidence that these hormones can also act synergistically in a dose- and kinetics-dependent manner (33, 39). Expression of PsABA was not affected by either treatment. However, PsPAL was significantly up-regulated in pheromone-exposed, egg-laden needles when sampled 12 d after pheromone treatment. Phenylalanine ammonia lyase catalyzes the biosynthesis of cinnamic acid, which is a precursor of numerous compounds formed along the phenylpropanoid pathway, among them compounds that contribute to plant cell wall lignification (36), which might impair larval hatching from D. pini eggs inserted into needle tissue.

Altogether, exposure of pine trees to sawfly sex pheromones affected the expression of several defense-related genes in a time-dependent manner after egg deposition (Table 1 and SI Appendix, Table S4). The combinatorial effects of pheromone exposure and subsequent egg deposition on the expression of PsSOD, PsLOX, PsPR-1, and PsPAL are striking. Hence, the pheromone exposure primes the enhanced expression of these genes in response to the sawfly’s egg deposition.

**Table 1. Expression of selected genes of P. sylvestris after exposure to sawfly sex pheromones and egg deposition**

<table>
<thead>
<tr>
<th>Time*</th>
<th>Hexane control†</th>
<th>Hexane + eggs</th>
<th>Pheromone</th>
<th>Pheromone + eggs</th>
<th>Significance† (P values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsRbah (Respiratory burst oxidase homolog – plant NADPH oxidase)</td>
<td></td>
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<tr>
<td>2d</td>
<td>1.00 ± 0.12ab</td>
<td>2.27 ± 0.71ab</td>
<td>3.07 ± 0.31ab</td>
<td>1.21 ± 0.13ab</td>
<td>0.001</td>
</tr>
<tr>
<td>12d</td>
<td>1.00 ± 0.13ab</td>
<td>0.19 ± 0.04ab</td>
<td>0.67 ± 0.20ab</td>
<td>0.43 ± 0.16ab</td>
<td>0.023</td>
</tr>
<tr>
<td>PsSOD (Superoxide dismutase)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>2d</td>
<td>1.00 ± 0.20</td>
<td>1.51 ± 0.32</td>
<td>1.01 ± 0.11</td>
<td>0.85 ± 0.09</td>
<td>0.254</td>
</tr>
<tr>
<td>12d</td>
<td>1.00 ± 0.10ab</td>
<td>0.70 ± 0.18ab</td>
<td>1.04 ± 0.14ab</td>
<td>1.62 ± 0.29ab</td>
<td>0.026</td>
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<tr>
<td>PsCAT (Catalase)</td>
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<tr>
<td>2d</td>
<td>1.00 ± 0.24ab</td>
<td>1.77 ± 0.33ab</td>
<td>1.23 ± 0.16ab</td>
<td>0.72 ± 0.12ab</td>
<td>0.048</td>
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<tr>
<td>12d</td>
<td>1.00 ± 0.20</td>
<td>0.60 ± 0.13</td>
<td>1.12 ± 0.26</td>
<td>1.36 ± 0.58</td>
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<td>PsAPX (Ascorbate peroxidase)</td>
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<tr>
<td>2d</td>
<td>1.00 ± 0.09</td>
<td>1.18 ± 0.18</td>
<td>0.89 ± 0.08</td>
<td>0.75 ± 0.06</td>
<td>0.112</td>
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<tr>
<td>12d</td>
<td>1.00 ± 0.16ab</td>
<td>0.40 ± 0.06ab</td>
<td>0.86 ± 0.10ab</td>
<td>1.26 ± 0.38ab</td>
<td>0.026</td>
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<td>PsLOX (Lipoxygenase)</td>
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<tr>
<td>2d</td>
<td>1.00 ± 0.29</td>
<td>0.80 ± 0.18</td>
<td>0.53 ± 0.19</td>
<td>1.00 ± 0.15</td>
<td>0.205</td>
</tr>
<tr>
<td>12d</td>
<td>1.00 ± 0.17ab</td>
<td>1.30 ± 0.24ab</td>
<td>0.92 ± 0.16ab</td>
<td>2.19 ± 0.31ab</td>
<td>0.015</td>
</tr>
<tr>
<td>PsPDF (Plant defensin)</td>
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<tr>
<td>2d</td>
<td>1.00 ± 0.42</td>
<td>0.49 ± 0.23</td>
<td>0.52 ± 0.21</td>
<td>0.47 ± 0.08</td>
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<td>12d</td>
<td>1.00 ± 0.27</td>
<td>0.93 ± 0.19</td>
<td>1.13 ± 0.19</td>
<td>1.22 ± 0.20</td>
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<tr>
<td>PsPR-1 (Pathogenesis related protein 1)</td>
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<tr>
<td>2d</td>
<td>1.00 ± 0.57</td>
<td>0.27 ± 0.10</td>
<td>0.43 ± 0.24</td>
<td>1.59 ± 1.08</td>
<td>0.384</td>
</tr>
<tr>
<td>12d</td>
<td>1.00 ± 0.29ab</td>
<td>3.29 ± 1.58ab</td>
<td>0.27 ± 0.19ab</td>
<td>6.13 ± 2.40ab</td>
<td>0.001</td>
</tr>
<tr>
<td>PsPAL (Phenylalanine ammonia lyase)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2d</td>
<td>1.00 ± 0.30</td>
<td>0.90 ± 0.47</td>
<td>0.73 ± 0.23</td>
<td>0.92 ± 0.18</td>
<td>0.654</td>
</tr>
<tr>
<td>12d</td>
<td>1.00 ± 0.23ab</td>
<td>1.66 ± 0.67ab</td>
<td>0.90 ± 0.22ab</td>
<td>3.43 ± 0.72ab</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Relative transcript abundance (mean ± SE) after treatment with pure hexane (the pheromone solvent; hexane control), with hexane and subsequent egg depositions (hexane + eggs), with pheromones dissolved in hexane only (pheromone), or with pheromone and subsequent egg deposition (pheromone + eggs); n = 5 to 8 trees for each treatment. Green highlights: transcript abundance significantly increased as compared to hexane control. Yellow highlights: transcript abundance significantly increased as compared to hexane control within a line: numbers in bold with different lowercase letters denote statistical differences (P ≤ 0.05).

*Days after start of pheromone exposure for 24 h; 2d = directly after 1 d of egg deposition.

†Expression values determined in untreated control trees did not differ from those in the “hexane control” (SI Appendix, Table S7).

‡Significance values (P) were calculated by Kruskal-Wallis H tests (compare SI Appendix, Table S4). Significant differences between 2 treatments were evaluated by a post hoc Conover-Iman test with a Benjamini-Hochberg correction for multiple comparisons.
have not been studied yet in pine (42). The costs of priming of with inducible ones and growth rates, possible costs of priming abundance of hungry larvae that will hatch from surviving eggs. larvae, because the greater egg mortality results in reduced vestment in later feeding-induced defense against hatching enhanced defensive response to the eggs might save costs of in- efficient way. These results suggest that such an early and en- ability to respond to insect pheromones allows a plant to resist them. Dubious population density despite the pine’s effective defense against them.
Another possibility of counteradaptation to the pheromone-mediated defenses of pine against D. pini eggs could be avoid- ance of oviposition on pheromone-exposed pine because of pheromone-induced oviposition-deterring changes in the need- les. Further studies are necessary to investigate this possible counteradaptation. Such a counteradaptation of an herbivorous insect to pheromone-primed defense against herbivory is sug- gested by results of the study of goldenrod plants exposed to male gall fly emissions; fewer oviposition punctures were de- tected in male-exposed plants than in control plants (3); how- ever, in this study, the survival of gall fly eggs and gall fly larval feeding upon the previously male-exposed plants could not be recorded. Nevertheless, these gall fly performance parameters are expected to be reduced because exposure of goldenrod plants to male gall fly emissions and their major component, con- ophthorin, primed the plants for improved defense against feeding damage by other goldenrod-specialized insects than the gall fly (3, 4).

Conclusion. Our study highlights that plant defense against eggs can be primed by an insect’s sex pheromone, which reliably in- dicates an impending very first step of plant infestation, the egg deposition. Hence, these findings show that a plant cannot only be primed for improved defense against impending feeding damage (3–11) but can even prepare its defense against insect eggs, which indicate impending larval feeding damage. Thus, the ability to respond to insect pheromones allows a plant to resist even the very beginnings of insect infestation, the eggs, in a more efficient way. These results suggest that such an early and en- hanced defensive response to the eggs might save costs of in- vestment in later feeding-induced defense against hatching larvae, because the greater egg mortality results in reduced abundance of hungry larvae that will hatch from surviving eggs. While constitutive defenses of pine have been shown to trade off with inducible ones and growth rates, possible costs of priming have not been studied yet in pine (42). The costs of priming of plant antiherbivore defenses—measurable by, for example, re- duced seed set, aboveground or belowground growth rate, and resistance against other biotic threats like phytopathogens—are considered to depend on various factors, among them the re- liability of the priming cue, the presence of priming-sensitive targets, and resource availability and competition (7, 10, 43–45).
Scots pine is shown here to improve its defense against insect eggs by responding to the insect’s sex pheromones with changes in the expression of its own defense-related genes and increased accumulation of egg-induced hydrogen peroxide. Our results provide the basis for further research addressing the questions arising here, such as about the specificity of the pine’s response to sawfly pheromones, the specificity of the response effects, and the perception of these pheromones. Components similar to the D. pini pheromonal compounds are released by closely related sawfly species. Females of other diprionidae genera than Diprion emit esters similar to the D. pini pheromonal esters, for example, esters with an alcohol component having a longer or shorter chain length than tridecanol or with other methylation patterns of tridecanol than in the D. pini pheromonal compounds (46).
The sawfly Diprion jingyuanensis, a pest of Chinese pine (Pinus tabulaeformis), has been shown to be attracted by the D. pini propionate pheromonal compound, suggesting that this is also a pheromone of D. jingyuanensis (47). Whether the Chinese pine species responds similarly to the pheromone and whether the eggs of D. jingyuanensis react similarly to the tree’s defense re- mains to be addressed in future studies. The lipophilic character of D. pini sex pheromones might facilitate direct interactions with the plant’s plasma membrane, and thus change trans- membrane ion fluxes and initiate early defense signaling (2). In addition to these proximate questions on the mechanisms in- volved, it will be interesting to address evolutionary ecology as- pects of this pheromone-mediated plant defense strategy. If the ability to respond to insect sex pheromones by priming defenses against insect eggs is widespread among plants, this might place some selective pressure on pheromone communication among insects and on their oviposition behaviors. Furthermore, if the priming effect shown by our study is not limited to the species studied here, but extends to other ones relevant in, for example,
agriculture and viticulture, application of the pheromone-mediated mating disruption technology in integrated insect pest management not only will cause negative effects on the fertilization of females due to olfactory insect disorientation (48) but will also reduce survival of insect eggs due to pheromone-primed plant defense.

Materials and Methods

Experimental Organisms. Three-year-old pine trees (P. sylvestris) were obtained from a tree nursery (Schlegel & Co.) and used for the experiments. The small trees were placed in Plexiglas cylinders (60 cm height, 9.5 litre), which were ventilated by charcoal-filtered air (inflowing and outflowing air: ~200 mL min⁻¹). As described above, the D. pini sex pheromones were dissolved in hexane; therefore, we also treated the trees with hexane only. Specifically, we used the following types of pine treatments (n = 4 per treatment analysis within several days after emergence from cocoons. No distinct mate calling behavior has been described for D. pini females, nor has it been observed by us. When we observed mating couples, they were sitting on the pine needles.

Plant Treatments. Prior to each experiment, trees from the greenhouse were acclimatized for 3 d in a climate chamber at 20 °C, 18% light:dark, 70% relative humidity, 155 μmol photons per square meter per second. To avoid cross-contamination with volatiles from plants that had been treated differently, the small trees were placed in Plexiglas cylinders (60 cm height, 9.5 litre), which were ventilated by charcoal-filtered air (inflowing and outflowing air: ~200 mL min⁻¹). As described above, the D. pini sex pheromones were dissolved in hexane; therefore, we also treated the trees with hexane only. Specifically, we used the following types of pine treatments (n = 4 per treatment analysis within several days after emergence from cocoons. No distinct mate calling behavior has been described for D. pini females, nor has it been observed by us. When we observed mating couples, they were sitting on the pine needles.

For treatment b, we applied 100 μL of a hexane solution to a cotton wool pad (diameter: 5.6 cm, thickness: 0.4 cm) as the dispenser. To allow evaporation of the pheromone components for 24 h and subsequent egg deposition for 24 h.

For treatment c, the plants were treated as in b, and, thereafter, 2 D. pini females were allowed to oviposit on the tree for 24 h. Only trees with at least 8 pheromone-exposed trees.

Determination of Egg Survival. To determine the effect of pheromone exposure on survival of leaves, we counted the number of eggs laid to the number of larvae hatching from the eggs per tree. Egg survival rates were determined on n = 6 untreated trees, n = 8 hexane-exposed trees, and n = 8 pheromone-exposed trees.

Determination of Pine Needle Water Content and Hydrogen Peroxide Concentration. To determine the water content of needles from the differently treated trees, we harvested 3 to 4 needles that were adjacent to the oviposition site. The needles were sampled 1) 2 d after pheromone or hexane exposure (i.e., 1 d after egg deposition) and 2) at the end of the egg incubation period, shortly before larvae would hatch, that is, 12 d after pheromone or hexane exposure and 11 d after egg deposition (egg incubation is around 12 to 14 d in the abiotic conditions used). Needles from equivalent positions and in comparable quantities were harvested from egg-free trees. Immediately after harvesting, the needles were weighed. The needles were then dried for 72 h in an oven (60 °C) and weighed once again. Based on these weight loss data, the relative water content (percent) was calculated. Drying for more than 72 h showed no further weight loss. We determined the water content of needles taken from n = 5 untreated trees and n = 8 trees subjected to the aforementioned treatments.

To determine the hydrogen peroxide concentrations of needles from the differently treated trees, we used the Amplex Red Hydrogen Peroxide/ Horseradish Peroxidase Assay Kit (Molecular Probes by Invitrogen), which provides a fluorescing product with hydrogen peroxide. Our protocol followed the manufacturer’s recommendations modified after Chakraborty et al. (54).

Needles were harvested from similar tree positions and at the same time points as described above for determining the water content. The needles were immediately transferred to liquid nitrogen after being detached from the experimental trees and were ground to a powder. A sample of 30 mg of powdered needle tissue per ml reaction mix was incubated with 10 μL of sodium phosphate buffer and placed on a shaker with 50 rpm at 25 °C for 30 min. Thereafter, the needle sample was centrifuged at 15,000 × g for 15 min, and the supernatant was centrifuged again at 15,000 × g for 2 min. A sample (50 μL) was taken from the final supernatant and incubated with 50 μL of a solution consisting of 100 μM Amplex Red reagent and 0.2 uL mL⁻¹ horseradish peroxidase. The incubation took 30 min at 30 °C in dark conditions. To prepare samples with distinct hydrogen peroxide concentrations for recording a reference standard curve, samples with hydrogen peroxide concentrations ranging from 0 to 30 μM H₂O₂ were prepared according to the protocol provided with the kit. These samples were incubated with the Amplex Red reagent and horseradish peroxidase as described for the needle samples. After incubation and centrifugation, the fluorescence of each sample (50 μL; 3 technical replicates) was determined by using an Infinite 200 PRO plate reader (Tecan Life Science) (excitation: 560 nm; emission: 590 nm). The hydrogen peroxide concentrations were calculated based on the standard curve value and then divided by 30 mg (needle sample weight). The hydrogen peroxide concentration was determined in needles taken from n = 8 trees of each treatment, as well as from n = 8 untreated trees.

Gene Expression Analysis. Needles were collected from sites adjacent to the oviposition site (about 1 g per tree) and from equivalent positions and in comparable quantities from egg-free trees. We harvested the needles at the same time points after pheromone exposure and egg deposition as described...
above for determining the water content. Needles that had been immedi-
ately frozen in liquid nitrogen after sampling were powdered. A powdered
needle extract (50 mg) was used for DNA extraction with the Invitrogen Spin
Plant RNA Mini Kit (Strategene). RNA was eluted in 50 μL of nuclease-free H2O,
and contaminating DNA remains were digested with the TURBO DNA free
kit (ThermoFisher Scientific). RNA integrity and purity were checked by
analysis on a 1.1% agarose gel in 1x TAE buffer with 0.006% EtBr. A volume
of 10 μL of the sample was diluted 1:1 with 2x RNAs loading dye (Thermo-
Fisher Scientific), heated for 10 min to 70 °C, and placed on ice immediately
afterward. A volume of 4 μL of the Ribolifter High Range RNA Ladder
(ThermoFisher Scientific) was treated likewise. After loading samples, the gel
was run for 90 min at 120 V. Spectrophotometric determination of the RNA
concentration was performed on a Multiscan GO microplate spectropho-
tometer (ThermoFisher Scientific) by measuring absorbance at 260 nm.

For synthesis of cDNA, 500 ng of extracted RNA was used as a template for
reverse transcription. A master mix for RT-PCR (avian myeloblastosis virus reverse
transcriptase) native enzyme (Roboklon). The RNA was mixed with 1 μL
of oligo dT20 (50 μM) and 2 μL of dNTPs (10 mM) and filled up to a reaction
volume of 14 μL with nuclease-free H2O. The mixture was incubated for
5 min at 65 °C, followed by 5 min incubation at 4 °C. To start the reaction, 4 μL
of 5x RT buffer (Roboklon), 0.5 μL of RNase inhibitor (Roboklon), 30 U
μL–1, 1 μL of 100 mM DTT (dithiothreitol), and 1 μL of AMV-RT native (Roboklon;
10 U μL–1) were added and heated to 42 °C for 15 min and then to 70 °C
for 45 min. To inactivate the AMV-RT enzyme, the mixture was finally heated
to 80 °C for 10 min and thereafter cooled on ice.

Primers (SI Appendix, Table S5) for the selected genes and for the house-
keeping genes ubiquitin (PsUBI), cytochrome subunit 6 (PsP6PET), and chloro-
plast ATPase beta subunit (PsATP) were designed and evaluated according to
the MIGE guidelines (minimum information for publication of quantitative
real-time PCR experiments) (55, 56) with the online tool named PRIMER-BLAST
(SF 57). For genes for which no annotated template sequences have been pub-
lished for P. sylvestris (LOX, PR-1; PETB; CATP; UB), we searched in BLAST (basic
local alignment search tool), EST (expressed sequence tags), and nr databases
for Pinus sequences, which showed high homology with annotated sequences
from other plant species. Primers were designed based on sequences with the
lowest E-value, and the identity of the PCR products was evaluated by Sanger
sequencing at Seqlab and BLAST analysis (SI Appendix, Table S6) (58).

We performed qPCR analyses using the qPCRbio SyGreen Mix Lo-Rox kit
(Nippon Genetics Europe) on an MX3005P (Strategene) cycler. For the qPCR
reactions, 12.5 ng of cDNA was mixed with 5 μL of qPCRbio SyGreen Mix Lo-Rox
Master Mix (Nippon Genetics Europe) and 0.17 μL of each primer (10 pmol μL–1)
and filled up to a 10-μL reaction volume with nuclease-free H2O. To control for
primer dimerization, H2O controls were run, and, to control for genomic DNA
contamination, DNase-treated RNA from each sample was used. Each reaction
was performed with 3 technical replicates under the following running condi-
tions: after an initial heating step of 2 min at 95 °C, 40 cycles of 5 s at 95 °C,
followed by 30 s at 60 °C, were performed. At the end of each cycle, the
fluorescence was measured twice. Following the 40 cycles of PCR amplification,
a dilution curve from 50 to 95 °C in 1 °C steps was performed to check for primer
dimer reaction products. Cq (cycle quantification value) and PCR efficiency of all
reactions were calculated with LinRegPCR version 2015.2.59 (SI Appendix).
Normalization of response genes to the reference genes PsUBI, PsP6PET, and
PsATP was performed as described by Vandesompele et al. (60). Gene expression
analyses were conducted with samples taken from n = 5 to 8 trees of each

Sawfly Antennal Responses to Pheromones. Electrophysiological antennal
responses of D. pini adult males and females to their sex pheromones
([25,3R,7R]-3,7-dimethyl-2-tridecanyl acetate and propionate) were recorded
by EAG. We chilled the sawflies by each sawfly at 4 °C for several minutes
and then cut off the antenna at its base, where we inserted the reference
electrode, that is, a glass electrode filled with Ringer solution (NaCl
128.3 mMol/L, KCl 4.7 mMol/L, CaCl2 2.6 mMol/L) and linked with a grounded
Ag wire. The tip of the antenna was connected to the windowed glass
electrode filled with Ringer as well and linked via an interface (IDAC 2;
Syntech) to a PC for signal recording. To record the electrophysiological
reactions, antennae were maintained in a humidity-controlled chamber
containing a 10 μL volume of the pheromone component, or 500 ng of the
proionate pheromone component, or 500 ng of each of the components as a blend on a filter paper
(28 mm2) (5 μL of pheromone solution in hexane; these quantities are
equivalent to that released by about 27 to 45 D. pini females). For control
measurements, 5 μL of hexane was applied to a filter paper. Prior to expo-
sure to the antenna, the solvent was allowed to evaporate for 15 min.
The signal of the filter paper with the test odor was dissected into a Pasteur
pipette, which was connected to a stimulus controller (CS-05; Syntech),
which allows puffing the test odor in a standardized manner to the antenna
(flow: 20 μl/s; stimulus time: 0.5 s). Each antenna was first exposed to ambient
air and then to one of the test odors or to the control solvent. The EAG
signals (millivolts) were amplified 100-fold by a microelectrode amplifier
and recorded by EAG software (Syntech). The EAG signals were evaluated by nor-
malizing the responses to test odors (R-t) to the responses to ambient air (R-a)
by dividing the signals (R-t/R-a). Likewise, the responses to the solvent hexane
(R-h) were normalized to those to air (R-h/R-a). Thereafter, the air-normalized
response to the solvent hexane was set to value 1.0 (R-h/R-a divided by R-h/R-a = 1),
and the air-normalized responses to the test odors were adjusted accordingly
(R-t/R-a divided by R-h/R-a). The signals recorded in response to the solvent
were almost the same as those in response to ambient air. We determined the
responses of n = 8 antennae (taken from 8 individuals) of each sex.

Data Analysis. The gene expression data were evaluated with the statistical
software R version 3.4.1 (61) using the packages car, lme4, and PMA. All
other data were evaluated with the statistical software SigmaPlot version
11.0 (Systat Software GmbH, 2008). All datasets were tested for normal
distribution by the Shapiro–Wilk test. Variance homogeneity was measured
with Levene’s test. Normally distributed data (with variance homogeneity)
were subjected to parametric tests, and nonnormally distributed data were
subjected to nonparametric tests. All tests (and respective P values) were run
2-sided with confidence intervals of 95%. To analyze the difference between
the recorded egg survival rates per pine treatment and the theoretically
possible survival rate (100% survival of all deposited eggs), we used the
paired t test. To analyze whether the survival rates, the water content, and
hydrogen peroxide concentrations differed among treatments, we used an
ANOVA, and, in the case of statistical significance, we further analyzed the
data by multiple pairwise t tests and a Benjamini–Hochberg P value

ACKNOWLEDGMENTS. We are grateful to Ute Braun, Beate Eisermann, and
Gabriele Haberberger, Freie Universität Berlin (FUB), for rearing the sawflies.
Many thanks are owed to Jona Hofflin, FUB, for his technical assistance and his
support in designing the primers. We are further grateful to the Collaborative
Research Centre 973 (Project B1, FUB) for its support. We also thank Helen Edlund
and Erik Hedenström from Mid Sweden University, Sundsvall, for the synthesis of
pine sawfly pheromones. A.A.-C. has been financially supported by the Eu-
erpean Research Council Synergy Grant ERC-SyG-2013-610028 IMBALANCE-P

and a grant from the German Academic Exchange Service (Grant 572142227).

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