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Research paper

Elm tree defences against a specialist herbivore are moderately primed by an infestation in the previous season

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The studies of the long-term effects of insect infestations on plant anti-herbivore defences tend to focus on feeding-induced damage. Infestations by an entire insect generation, including egg depositions as well as the feeding insects, are often neglected. Whilst there is increasing evidence that the presence of insect eggs can intensify plants' anti-herbivore defences against hatching larvae in the short term, little is known about how insect infestations, including insect egg depositions, affect plant defences in the long term. We addressed this knowledge gap by investigating long-term effects of insect infestation on elm's (*Ulmus minor* Mill. cv. 'Dahlem') defences against subsequent infestation. In greenhouse experiments, elms were exposed to elm leaf beetle (ELB, *Xanthogaleruca luteola*) infestation (adults, eggs and larvae). Thereafter, the trees cast their leaves under simulated winter conditions and were re-infested with ELB after the regrowth of their leaves under simulated summer conditions. Elm leaf beetles performed moderately worse on previously infested elms with respect to several developmental parameters. The concentrations of the phenylpropanoids kaempferol and quercetin, which are involved in egg-mediated, short-term effects on elm defences, were slightly higher in the ELB-challenged leaves of previously infested trees than in the challenged leaves of naïve trees. The expression of several genes involved in the phenylpropanoid pathway, jasmonic acid signalling, and DNA and histone modifications appeared to be affected by ELB infestation; however, prior infestation did not alter the expression intensities of these genes. The concentrations of several phytohormones were similarly affected in the currently challenged leaves of previously infested trees and naïve trees. Our study shows that prior infestation of elms by a specialised insect leads to moderately improved defences against subsequent infestation in the following growing season. Prior infestation adds a long-term effect to the short-term enhancer effect that plants show in response to egg depositions when defending against hatching larvae.

Keywords: egg deposition, elm leaf beetle, epigenetic marks, flavonoids, phytohormone, plant defence.

Introduction

In forests, mass outbreaks of insect pests can cause severe damage and even the mortality of trees (Anderegg et al. 2015, Fei et al. 2019). However, trees have evolved a wide range of constitutive and infestation-inducible defences to help them cope with a multitude of herbivorous insect species (Haukioja 1990, Mumm and Hilker 2006, Eyles et al. 2010, Holopainen 2011, Bräutigam et al. 2013). Moreover, trees can improve their defences against insect herbivory after exposure to environmental cues that are indicative of impending insect infestation. Previous herbivory (Tscharntke et al. 2001), exposure to leaf volatiles from damaged neighbouring trees (Tscharntke et al. 2001, Frost et al. 2008, Brosset and Blande 2022), and perception of insect egg depositions on leaves (Beyaert et al. 2012) can all significantly improve a tree's response to subsequent herbivory.

Previous herbivory exerts both short- and long-term effects on plant defences against subsequent herbivory (Haukioja et al. 1985, Roitto et al. 2009, Rasmann et al. 2012, Mertens et al. 2021, Sobral et al. 2021). The long-term effects may last for months and even persist over several growing seasons (Clausen et al. 1991, Zvereva et al. 1997, Ruuhola et al. 2007). Studies

on the long-term effects of insect infestation on plant defences against subsequent infestation usually test the effect of leaf damage, i.e. herbivory or leaf wounding, but have neglected insect egg depositions, which precede feeding damage by the hatching larvae (Karban 1990, Bryant et al. 1991, Rasmann et al. 2012, Sobral et al. 2021).

Insect egg depositions are known to exert short-term effects on plant defences. Both herbaceous and perennial plant species responding to previous insect egg deposition show improved defences against hatching larvae (Hilker and Fatouros 2015, Pashalidou et al. 2015, Austel et al. 2016, Bandoly et al. 2016, Hilker and Fatouros 2016, Rondoni et al. 2018, Lortzing et al. 2019, Valsamakis et al. 2020, Valsamakis et al. 2022). Egg-mediated plant responses to larval herbivory are associated with specific transcriptional, phytohormonal and metabolite responses to larval feeding damage. For example, *Arabidopsis thaliana* leaves laden with *Pieris* butterfly eggs showed an enhanced expression of genes involved in salicylic acid (SA) and jasmonic acid (JA) biosynthesis and signalling (Bruessow et al. 2010, Valsamakis et al. 2020). The concentrations of SA, JA-isoleucine (JA-Ile) and abscisic acid (ABA) were enhanced in egg-laden, feeding-induced plants when compared with egg-free, feeding-induced plants (Valsamakis et al. 2020). Chemical analyses of the feeding-damaged leaves of several plant species have shown that the concentrations of phenylpropanoids are significantly higher in previously egg-laden leaves than in previously egg-free leaves (Lortzing et al. 2020 and references therein).

It is currently unknown whether the short-term effects of insect egg deposition on plant defences may be further enhanced by a prior infestation. We aimed to address this knowledge gap by studying the long-term effects of a first insect infestation on egg-mediated plant defences against a subsequent infestation in the following growing season.

We used elm (*Ulmus minor*) and the elm leaf beetle (ELB, *Xanthogaleruca luteola*) for our study. Elm is known to show short-term responses to ELB egg deposition that improve its resistance against hatching ELB larvae (Austel et al. 2016). A comparison of the responses of egg-laden and egg-free elm leaves to ELB feeding damage showed that the egg-laden leaves contained higher concentrations of the phenylpropanoids kaempferol and quercetin, and higher concentrations of SA and the transcript levels of *PAL* (phenylalanine ammonia lyase), a gene at the entrance of the phenylpropanoid pathway (Schott et al. 2022). The performance of ELB larvae on elm leaves treated with a high concentration of a flavonoid was weaker than on untreated leaves (Austel et al. 2016). Another analysis of the transcriptomic responses of elm to ELB infestation showed a high number of differentially expressed genes in response to egg deposition (Altmann et al. 2018). However, shortly before larval hatching, i.e. 7 days after egg deposition, the differential expression of almost all egg-responsive genes reverted to their control levels. In response to larval feeding damage, the previously egg-laden leaves showed earlier, more differentially expressed genes than the egg-free leaves, suggesting (i) an egg-mediated acceleration of the transcriptomic response to feeding damage and (ii) a 'memory' of the response to eggs by a subset of genes (Altmann et al. 2018). It is currently unknown whether this 'memory' effect is due to egg-induced epigenetic changes. In contrast to our limited understanding of the impact of insect egg depositions on the epigenome, there is good evidence to show that insect herbivory causes significant changes in the plant's epigenetic marks (DNA methylation and histone modification) (Alonso et al. 2019, Annacondia et al. 2021, Sobral et al. 2021), which may affect the plant's responses to subsequent feeding damage (Rasmann et al. 2012).

To elucidate whether the short-term effects of ELB egg deposition on plant defences are further enhanced by an ELB infestation in the previous growing season, we first studied the ecological effects of a prior infestation on elm defences against ELB and then characterised the physiological and molecular effects of this infestation. We addressed in detail the following questions: (i) How does a first ELB infestation (including eggs, larvae and adults) affect the performance of ELB larvae and adults that develop on regrown, egg-treated leaves in the following season? (ii) Do previously infested trees receive a lower number of ELB eggs in the following season than naïve trees? (iii) Do concentrations of the phenylpropanoids kaempferol and quercetin differ between the currently infested elm leaves of previously infested trees and the leaves of naïve trees? (iv) Does a prior elm infestation by ELB (eggs, larvae and adults) affect the concentrations of phytohormones and the expression of genes involved in phenylpropanoid and phytohormone biosynthesis, signalling and epigenetic modifications (DNA methylation and histone modifications) in leaves currently exposed to eggs and larvae?

Materials and methods

Plants and insects

All experiments were conducted with elm trees derived from a clonal culture of *U. minor* Mill. cv. 'Dahlem'. The in vitro shoot culture was maintained on enriched DKW medium with 0.01 mg l^{-1} indole-3-butyric acid (IBA, Sigma-Aldrich, St. Louis, MO, USA) and 1 mg l^{-1} 6-benzylaminopurine (BAP, Sigma-Aldrich) as explained in detail by Büchel et al. (2011). For rooting, 2- to 4-cm-long shoots were transferred to the enriched DKW medium at half the concentration and containing 3 mg l^{-1} IBA hormone but no BAP. After ~1 week, the shoots were transferred to full-strength DKW medium without phytohormones. Plants that developed roots were then transferred to soil and kept in a climate chamber at 25 °C and under 16-h light until they were ~6 months old.

Four weeks before the experiments started, 20 6-month-old trees were potted in 5-l pots and transferred to the greenhouse,

where they were kept at 25 °C (day)/20 °C (night) and provided with additional light for 16 h during the daytime (EYE IWASAKI MT 400 W/DL, Iwasaki electric Co. Ltd, Tokyo, Japan). One week before the experiment started, the trees were transferred to individual mesh cages (BugDorm-2 Medium Insect Rearing Tent Fine Nylon Mesh, 75 cm × 75 cm × 115 cm, MegaView Science, Taichung, Taiwan). The temperature was reduced to 23 °C (day)/18 °C (night). Plants were exposed to additional light for 14 h during the daytime.

Elm leaf beetles were reared on *U. minor*. The rearing was refreshed every year with beetles collected in the field in France, close to Montpellier, where this species occurs in high population densities. Adult beetles were kept in micro-perforated polypropylene bags on the twigs of potted elm trees at room temperature (22–25 °C, 16 h light during daytime with 625–800 lux, 65–75% relative humidity). Beetles fed, mated and laid eggs upon the leaves in those bags. Twigs with egg-laden leaves were enclosed in fresh, micro-perforated bags, and the adults were transferred to another twig. The larvae hatching from eggs remained confined in the bags. When the larvae had consumed most of the leaf material within a bag, twigs with larvae were placed into plastic boxes covered with a gauze lid. The larvae were provided with fresh elm twigs three times a week until they pupated. Pupae were stored on paper towel in plastic boxes covered with a gauze lid until the adults emerged.

General experimental design

The experiments were conducted in our greenhouse. We investigated how elms that had been previously infested by ELB adults, eggs and larvae during (simulated) summer and autumn conditions respond to a subsequent ELB infestation occurring after (a simulated) winter on newly grown leaves (Figure 1). The simulated seasonal light and temperature conditions in the greenhouse corresponded to those in Central Europe. An overview of the abiotic conditions under which the elm trees were kept prior to the experiments and during the experiments is provided in Table S1 available as Supplementary data at *Tree Physiology* Online.

We performed two experiments. The first was conducted to compare insect performance on previously ELB-infested trees and naïve trees. The second experiment provided leaf material for analysis of the chemical, phytohormonal and transcriptional responses of ELB-infested trees that (i) had been infested in a prior growing season and (ii) had not been exposed to a previous ELB infestation. We could not use the trees from the first experiment for leaf material analysis since we did not want to artificially damage the trees while they were being monitored for insect performance.

Plant treatments

For each experiment, we treated (i) n = 10 trees with an initial infestation and a second infestation, after a winter period. We refer to these trees as I-EF trees [previously infested by an entire leaf beetle generation and subsequently exposed again to eggs and (larval) feeding damage]. (ii) Another 10 trees were exposed to only a single infestation, which corresponded to the second infestation of the I-EF trees. We refer to these trees as EF trees [exposure to egg depositions and (larval) feeding damage] (Figure 1). Treatments were assigned randomly to the trees, which were spaced ∼80 cm apart from each other in the greenhouse.

The first infestation of the I-EF trees was conducted under simulated summer and autumn conditions. We transferred 15 randomly selected, 1- to 7-day-old adult ELB (equal mix of both sexes by a visual inspection of the beetles) onto 7-month-old *U. minor* trees (∼90 cm in height), which were enclosed in a mesh cage 1 week before, as explained in the section 'Plants and insects'. The adults mated and fed upon the leaves, and females laid their egg clutches on the underside of the leaves. All hatching larvae fed on the trees until pupation. Pupae matured to adults, which were also allowed to further infest the trees.

Then, we stepwise subjected the trees to simulated winter conditions (see Table S1 available as Supplementary data at *Tree Physiology* Online). Elm leaf beetle larvae and beetles were active for 8 weeks before they ceased to feed due to decreasing light and temperature conditions. By this time, approximately one-third of the leaves on each tree had been damaged by feeding. Approximately 11 weeks after the start of the experiment, the trees had cast all their leaves (Figure 1). Inactive and dead insects were removed together with the cast-off leaves.

Following a simulated winter period of 9 weeks, we gradually increased day length and temperatures over 8 weeks until they had returned to summer conditions. Approximately 20 weeks after the start of the experiment (Figure 1), the trees began to flush under spring conditions.

The EF trees were kept at the same conditions as the I-EF trees but were not exposed to any ELB infestation up to this time.

The second ELB infestation—consisting of an egg deposition cue and larval feeding—was initiated 28–32 weeks after the first infestation had started, i.e. when the new foliage was fully developed (Figure 1). Since we could not infest all trees at once due to the limited availability of gravid females and neonate larvae, we infested the trees sequentially. The pairs of EF and I-EF trees were always treated in parallel.

We applied six 'standardised ELB egg depositions' (SEDs) per tree as described by Austel et al. (2016), Altmann et al. (2018) and Schott et al. (2022). This standardised infestation allowed us to compare our results with those of our previous studies, which addressed the impact of ELB egg deposition on short-term responses to larval feeding among trees that had not previously been infested (Austel et al. 2016, Altmann et al. 2018, Schott et al. 2022). In the present study, we compared how the responses of EF-treated trees differed from those of I-EF-treated trees that had been infested by an entire ELB

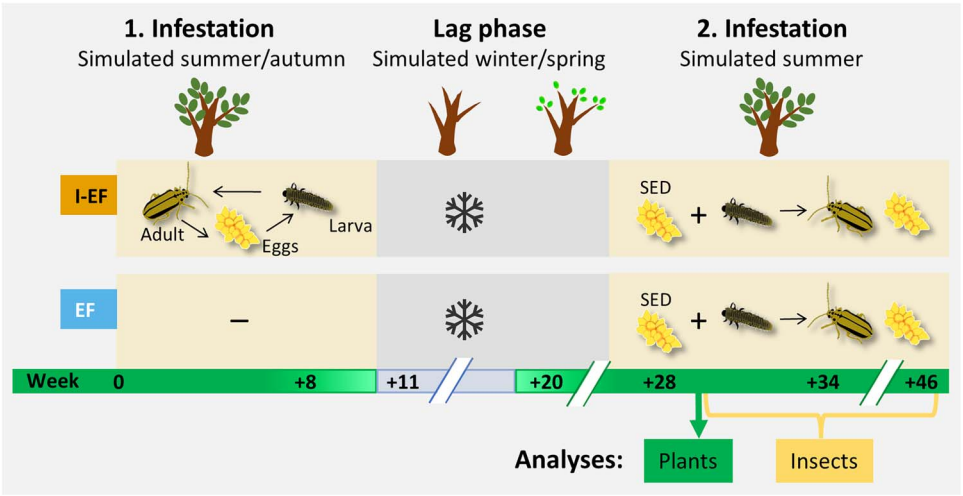

Figure 1. General experimental design. Young *U. minor* trees were exposed in the greenhouse to infestation by the elm leaf beetle *X. luteola.* Across 46 weeks, the trees were kept under various abiotic conditions simulating European seasons. Detailed information on the temperature and light conditions are provided in Table S2 available as Supplementary data at *Tree Physiology* online. I-EF trees were exposed to two infestations; during the first, each tree was exposed to 15 adults, their egg depositions, feeding damage by larvae hatching from the eggs and the subsequent developing elm leaf beetle population. The second infestation occurred after a simulated winter period. The regrown leaves of the trees were exposed to 'standardised egg depositions' (SEDs) (six per tree) and subsequently to 30 neonate larvae per tree. EF trees were exposed to only a single infestation, carried out as described for the second infestation of the I-EF trees (above). Analyses 'plants': elm leaves were sampled from I-EF and EF trees after 1 day of feeding by neonate larvae; we analysed the concentrations of phenylpropanoids, phytohormones and gene expression levels in these leaves. Analyses 'insects': we monitored parameters of insect development and performance during the second infestation period (the duration of larval development; the weight of 8-day-old larvae, pupae and adults; the mortality of 8-day-old larvae; the percentage of adults successfully emerging from pupae; and the number of eggs laid by adults). The insect performance data and plant data were obtained in separate experiments, each with 9–10 elm trees per treatment.

generation in the previous growing season. For applying SEDs, we gently removed a tiny piece of the abaxial leaf epidermis with a scalpel and immediately covered this site with oviduct secretion from the *oviductus communis* of a freshly killed, gravid female beetle. The *oviductus communis* of one female provided oviduct secretion for two SEDs. This method is known to elicit a plant response similar to that observed after natural egg deposition (Meiners and Hilker 2000, Altmann et al. 2018). Seven days later, after the natural period for egg incubation, five neonate larvae from our rearing were carefully placed with a smooth brush on each SED-treated leaf (i.e. 30 larvae per tree) and confined in a clip cage. The neonate larvae that were placed on one replicate of an EF and I-EF tree were from the same pool of freshly hatched larvae.

For the first experiment that was designed to analyse ELB performance, we proceeded as follows: after 4 days, larvae were transferred to neighbouring leaves for four more days. They were then enclosed in three groups in micro-perforated polypropylene bags on the twigs of the same tree. Prepupae were collected from the bags and transferred separately in 2-ml tubes (Eppendorf, Hamburg, Germany), where they pupated and adults emerged. We randomly selected four females and three males from those adult ELBs and placed them back on an undamaged twig of the tree where they had spent their juvenile development. The twig was enclosed in a micro-perforated polypropylene bag. When the leaf material of the twig had been consumed, the beetles were placed on a new, undamaged twig of the same tree to provide them with fresh leaf material. The adults fed, mated and laid eggs on those twigs for 9 weeks. We did not place all females that had successfully developed into adults onto the trees because they did not have enough leaf material to provide all beetles with fresh material for the duration of the study. At week 46, the first experiment was finished (Figure 1).

The second experiment that was designed to analyse leaf parameters was performed in the same way as the first one. However, it was completed already at week 29–33, soon after the second infestation was initiated (Figure 1).

Performance of elm leaf beetles

In the first experiment, we determined the weight and mortality rate of the larvae of the second infestation after an 8-day feeding period on EF and I-EF trees (10 trees with up to 30 larvae per treatment). We also recorded the time until larvae pupated, as well as the weight of pupae and freshly emerged adult beetles. In addition, we determined the percentage of adult beetles that successfully emerged from pupae (Figure 1).

To compare the ELB egg load that I-EF and EF trees needed to cope with after an ELB infestation of one generation, the number of eggs laid by the four ELB females per tree was recorded after 3, 6 and 9 weeks. Collecting eggs during different time intervals allowed us to check for differences in the numbers of

eggs during the early, intermediate and late phase of egg laying. We calculated the number of eggs which might be expected on these I-EF and EF trees when taking into account the number of all ELB females that successfully developed into adults on those trees. To this end, we first divided the total number of eggs on a tree laid by the four females by four and then multiplied this by the number of females that successfully developed into adults on the respective tree.

Leaf sampling for analysis of chemical, phytohormonal and transcriptional responses to treatments

In the second experiment, we harvested locally treated leaves from EF and I-EF trees during the second infestation after a larval feeding period of 1 day (Figure 1, $n = 10$ per treatment). At the same time, we sampled untreated leaves from branches below the treated branches of both EF trees (C_{EF} samples) and I-EF trees ($C_{I\text{-}EF}$ samples). We know from our previous studies that elm responses to a brief ELB larval feeding period of 1 day are mostly restricted to the locally damaged leaves (Schott et al. 2022). The leaf samples were frozen immediately in liquid nitrogen and stored at −80 °C. The aliquots of homogenised leaf material were ground to a fine powder under liquid nitrogen and stored at −80 °C until they were needed for further analyses.

Concentrations of total kaempferol and quercetin

To determine the concentrations of kaempferol and quercetin (including their *O*-bond derivatives) in feeding-damaged EF and I-EF leaves, as well as in the undamaged (untreated) C_{EF} and $C_{I\text{-}EF}$ leaves of the second experiment, methanolic leaf extracts were prepared and subjected to an acidic hydrolysis. In this way, *O*-bond derivatives were cleaved off, leaving the kaempferol and quercetin core structures for analysis by high-performance liquid chromatography - diode-array detection (HPLC-DAD), as described by Schott et al. (2022). In short, a 50-mg aliquot of finely ground leaf powder was extracted twice with 750 μl of 80% methanol. We added 5 μg of umbelliferone as an internal standard and yielded 1,300 μl of crude leaf extract. An aliquot of 700 μl of each of these crude elm leaf extracts was subjected to an acidic hydrolysis with 1.2-M HCl according to a modified protocol from Hertog et al. (1992) and Mattila et al. (2000), as described in Schott et al. (2022). The resulting residue was dissolved in 200 μl of 70% methanol; 10 μl of this was analysed using HPLC-DAD.

The HPLC-DAD analysis was performed on a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) with a diode array detector (SPD-M20A). Separation was performed using an Intersil ODS-3 column (4.6 mm × 150 mm, 3-μm particle size, pre-column 4.6 mm × 15 mm, Intersil Corp., Milpitas, CA, USA). We used 0.25% phosphoric acid in water as eluent A and acetonitrile as eluent B, with a linear increasing flow gradient from 0 to 80% (for details, see Schott et al. 2022). Umbelliferone was monitored at 324 nm (Rt 14.72 min), kaempferol at 265 nm (Rt 20.72 min) and quercetin at 255 nm (Rt 18.65 min). The peak areas of kaempferol and quercetin were normalised to the internal standard umbelliferone. The concentrations of kaempferol and quercetin in leaf material (in ng per gram fresh weight) were calculated using external calibration curves with authentic reference compounds (Sigma-Aldrich).

Phytohormone concentrations

We determined the concentrations of the phytohormones SA, JA, JA-Ile and ABA in EF and I-EF leaves, as well as in the respective downstream C_{EF} and $C_{I\text{-}EF}$ leaves in the second experiment. The phytohormone extraction was carried out following a modified protocol from Wang et al. (2007) and as described by Schott et al. (2022). Briefly, 80–100 mg (fresh weight) leaf material was extracted twice with ethyl acetate by homogenisation and subsequent centrifugation. In the first extraction step, deuterated phytohormones were added as internal standards (20 ng of D4-SA and 20 ng of D6-ABA, both from OlChemIm Ltd, Olomouc, Czech Republic, as well as 20 ng of D6-JA and 60.4 ng of D6-JA-*L*-Ile, both from HPC Standards GmbH, Cunnersdorf, Germany). The supernatants were combined, evaporated at room temperature to a honey-like viscosity and then dissolved in 400 μl of 70% methanol with 0.1% formic acid. The extracts were stored at −20 °C, until needed for analysis.

The phytohormone analysis of 7 μl of the particle-free extracts was performed using UPLC-ESI-MS/MS (Q-ToF-ESI) on a Synapt G2-S HDMS (Waters®, Milford, MA, USA). Separation was performed on a C_{18} column (Acquity UPLC Waters, BEH-C18, Ø 2.1 mm × 50 mm, particle size 1.7 μm) with water and methanol [each with 0.1% formic acid (v/v)] (for details, see Schott et al. 2022). Compounds were detected by tandem mass spectrometry scanning the full mass spectrum of compounds between 50 and 600 *m/z*.

Phytohormones were annotated according to their parent $[M\text{-}H]^-$ ion and a diagnostic daughter ion [SA (*m/z* 137 and 93), ABA (*m/z* 263 and 153), JA (*m/z* 209 and 59) and JA-Ile (*m/z* 322 and 130)], as well as according to co-elution with their deuterated derivatives of the internal standard [D4-SA (*m/z* 141 and 97), D6-ABA (*m/z* 269 and 159), D6-JA (*m/z* 215 and 59) and D6-JA-Ile (*m/z* 328 and 130)]. For quantification, the peak area of the daughter ions of the natural phytohormones was related to the peak area of the internal standard's daughter ions using MassLynx™ Software (version 4.1, Waters®). Concentrations per sample were normalised according to the fresh weight of the leaf material analysed.

qPCR analysis

We determined the expression levels of certain marker genes that are either known to respond to the presence of insect eggs followed by larval feeding or that have been hypothesised to play a role in these responses (see Introduction and references

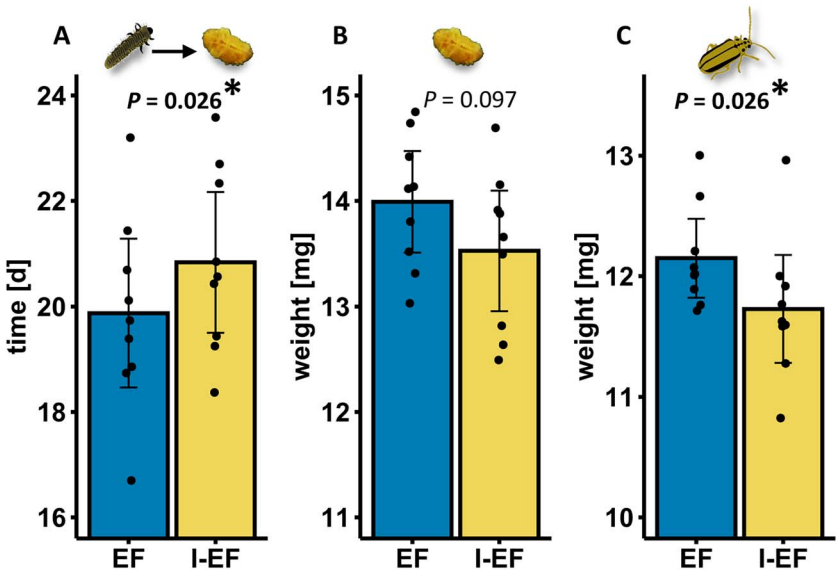

Figure 2. Performance of *X. luteola* on *U. minor* trees that had been infested by *X. luteola* in the previous growing season (I-EF) or that had not been exposed to a prior infestation (EF). (A) Development time of larvae from hatching until pupation in days, the weight of (B) pupae and (C) adult beetles in mg. Bars represent mean ± 95% CI of $n = 9$ replicates. Paired *t*-test; bold *P*-values marked by an asterisk: significant results ($P < 0.05$).

in this paragraph). We ran the qPCR analyses of EF, I-EF, C_{EF} and $C_{I\text{-}EF}$ leaves and compared the expression levels of sequences homologous to genes involved in the phenylpropanoid pathway (*PAL*, *phenylalanine ammonia lyase*; *ANS*, *leucoanthocyanidin dioxygenase*), in JA signalling (*13-AOS*, *allene oxide synthase*; *JAZ 10*, *jasmonate ZIM domain protein 10*) and in histone and DNA modifications (*HDA19*, *histone deacetylase 19* (Zhou et al. 2005, Kim et al. 2008, Choi et al. 2012, Wasternack and Hause 2013); *JMJ13* and *JMJ27*, *JUMONJI13* and *27* (histone demethylases) (Li et al. 2013, Dutta et al. 2017, Zheng et al. 2019, Keyzor et al. 2021, Wang et al. 2021); and *DME*, *transcriptional activator DEMETER* (a DNA demethylase) (Kellenberger et al. 2016, Latzel et al. 2020, Zeng et al. 2021).

Total RNA was extracted from 50–60 mg leaf powder according to a chloroform-based protocol modified from Ikoma et al. (1996) and Altmann et al. (2018), as described in Schott et al. (2022). The protocol includes the addition of polyvinylpolypyrrolidone to reduce disturbance by leaf polyphenols and an extraction step with ethanol, potassium acetate and chloroform:isoamyl alcohol to remove polysaccharides. The pellet was dissolved in nuclease-free water and frozen at −80 °C until further processing. Residual DNA was removed using DNase (DNA-free™ Kit, Thermo Fisher Scientific, Waltham, MA, USA). The concentration and purity of RNA were determined spectrophotometrically, and RNA integrity was checked by gel electrophoresis on 1.8% agarose gels.

First-strand cDNA was synthesised from 1 μg total RNA with the RevertAid™ RT Reverse Transcription Kit (Thermo Fisher Scientific) following a modified protocol using oligo-dT and random hexamers to promote reverse transcription. Primer sequences for phenylpropanoid biosynthesis–related genes were adopted from Schott et al. (2022). Homologues to genes involved in DNA and histone modification and JA-related genes were identified by blasting annotated sequences from *A. thaliana* to an elm RNA-seq data set from Altmann et al. (2018). Primers were designed using Primer3web version 4.1.0 (Untergasser et al. 2012) for the sequences listed in Table S2 available as Supplementary data at *Tree Physiology* Online. Each primer pair was tested for amplification efficiency and specificity by melting curve analysis and gel electrophoresis on a 2% agarose gel.

Quantitative real-time PCR (qRT-PCR) was performed in technical triplicates with 10-μl reaction volumes containing 10-ng cDNA and 5 μl of Power SYBR© Green Master Mix (Thermo Fisher Scientific) on a CFX96 Real-Time System with a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a thermal profile of 1 × 95 °C for 10 min, 40 × 95 °C for 20 s and 1 × 60 °C for 60 s, followed by a melting curve analysis (60–95 °C). The transcript levels of target genes were normalised to sequences homologous to the SAND family gene, UBQ (polyubiquitin) and Splicing factor3B subunit 5-like, which showed a stable expression in a previous elm study by Altmann et al. (2018) (for primer sequences, see Table S3 available as Supplementary data at *Tree Physiology* Online). In order to quantify the expression levels of our genes of interest, we calculated a reference gene index by determining the geometric mean of the expression levels of the three reference genes (Vandesompele et al. 2002). The relative expression levels of each gene of interest were calculated by relating the determined $2^{-\text{deltaCt}}$ values to the reference gene index according to a modified protocol by Livak and Schmittgen (2001).

Statistics

Statistical analyses and data visualisations were carried out with the software 'R' (version 4.0.2, R Core Team 2020), using

the packages car (version 3.0-10, Fox and Weisberg 2019), ComplexHeatmap (version 2.4.3, Gu et al. 2016, http://bioconductor.org/biocLite.R), egg (version 0.4.5, Auguie 2019), ggplot2 (version 3.3.5, Wickham 2016), lme4 (version 1.1–27, Bates et al. 2015), multcomp (version 1.4–17, Hothorn et al. 2008), plyr (version 1.8.6, Wickham 2011) and rstatix (version 0.7.0, Kassambara 2021).

Normal distributions of data and their variance homogeneity were inspected with Q-Q plots (Wilk and Gnanadesikan 1968) and the Shapiro–Wilk and Levene's tests. Data not normally distributed were $\log_2$-transformed and inspected again. For flavonoid and phytohormone data, extreme outliers [values above Q3 + 3 × interquartile range (IQR) or below Q1–3 × IQR (Tukey 1977)] were excluded from further analyses. Replicate 10 of the first experiment was excluded from analyses because the respective I-EF tree showed abnormal, crippled growth with small, senescent leaves with low chlorophyll content.

The data for the weight and developmental times of individual ELB feeding on the leaves of paired EF and I-EF trees were compared by paired *t*-tests. Elm leaf beetle mortality data were evaluated by general linear models (GLMs). We compared the calculated number of ELB eggs per EF and I-EF tree per time interval using the paired Student's *t*-test or Wilcoxon signed-rank test (depending on data normality). Treatment effects on the number of eggs laid on I-EF and EF trees across the time intervals were analysed using a negative binomial GLM, with treatment and time interval as fixed factors with an interaction term and replicate as a random factor (family: Quasi-Poisson since there was overdispersion using Poisson distribution). We checked the significance of fixed factors using a chi-square test.

We compared flavonoid and phytohormone concentrations in EF, I-EF, C_{EF} and $C_{I\text{-}EF}$ leaves using an ANOVA and Tukey's test as post hoc tests, or the Welch ANOVA and Games–Howell test if the homogeneity of variances was not satisfied. The expression levels of genes in EF, I-EF, C_{EF} and $C_{I\text{-}EF}$ were compared using Kruskal–Wallis tests and pairwise Wilcoxon rank-sum tests with the Benjamini–Hochberg correction as a post hoc test.

Results

Herbivore performance is moderately worse on previously infested I-EF trees than on EF trees

Elm leaf beetle larvae that fed on previously infested I-EF trees were moderately impaired in their development compared with those feeding on EF trees that had not been infested before. Larval development time from hatching until pupation was significantly prolonged by ∼1 day (Figure 2A). Despite the longer larval development time, the resulting pupae tended to gain less weight on previously infested trees, whereas the resulting beetles showed significantly lower weight when having spent their juvenile development on I-EF trees than on EF trees (Figure 2B and C). However, the mortality of 8-day-old larvae and the percentage of beetles, which successfully emerged from pupae, were similar for individuals on I-EF and EF trees (see Table S4 available as Supplementary data at *Tree Physiology* Online).

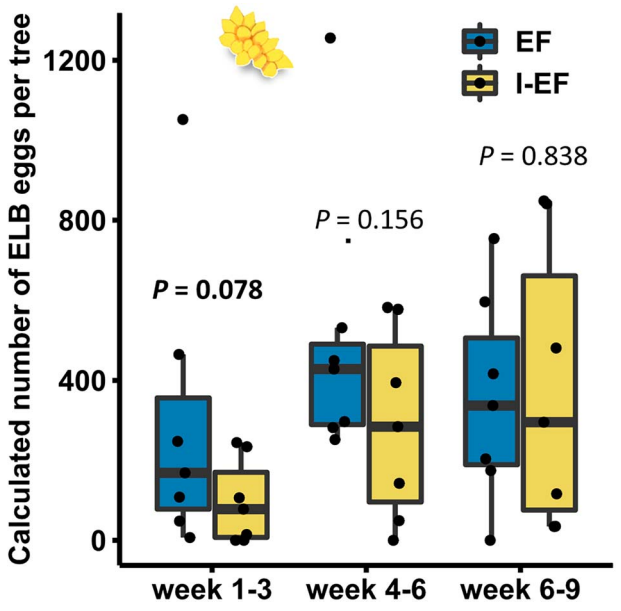

Figure 3. Calculated number of eggs laid by *X. luteola* on *U. minor* trees that had been infested by *X. luteola* in the previous growing season (I-EF) or that had not been exposed to a prior infestation (EF). Eggs were laid by females that had spent their juvenile development on those same trees. For the details of these calculations, see Materials and methods. The Wilcoxon signed-rank test was used to analyse for differences between treatments during single time intervals. Boxplots represent median, first and third quartiles of $n = 7$ replicates. Boxplot whiskers include data within the first quartile −1.5 interquartile range (IQR) and third quartile +1.5 interquartile range. *P*-value < 0.1 in bold.

Previously infested I-EF trees receive an egg load similar to that of EF trees

When considering the total number of eggs laid during the 9-week oviposition period, we did not detect significant differences (i) between the number of eggs laid by a female on I-EF and EF trees (mean ± SE of eggs per one female on the EF tree: 135 ± 31; I-EF tree: 116 ± 28); or (ii) between the calculated number of eggs potentially laid on an I-EF and EF tree by all females that had successfully developed per tree (mean ± SE of calculated # eggs on the EF tree: 1154 ± 335; I-EF tree: 766 ± 207, see also Table S5 available as Supplementary data at *Tree Physiology* Online). An analysis of the data using a negative binomial GLM did not find the elm treatment (I-EF and EF) to have a significant influence on the number of eggs laid across the three time intervals, i.e. the treatment × time interval interaction term was not significant (see Table S6 available as Supplementary data at *Tree Physiology* Online).

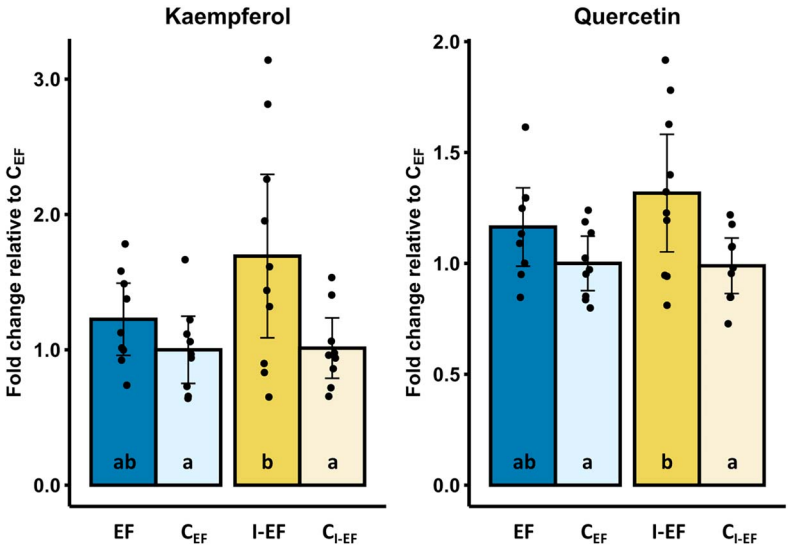

Figure 4. Impact of *X. luteola* infestation on flavonoid concentrations in *U. minor* leaves. Total concentrations of kaempferol and quercetin were analysed in hydrolysed methanolic leaf extracts. EF = leaves with eggs and 1 day of larval feeding upon trees without prior infestation. I-EF = leaves with eggs and 1 day of larval feeding upon trees that were exposed to a *X. luteola* infestation in the previous growing season. C_{EF}, C_{I-EF} = untreated control leaves located below EF and I-EF leaves on the same trees. Bars represent mean ± 95% CI of fold-change of concentration relative to leaves on untreated branches of EF trees (C_{EF}). The concentrations of kaempferol and quercetin were compared by the ANOVA and, in the cases of a significant result ($P < 0.05$), with the Tukey test (different letters indicate significant differences at $P < 0.05$). Kaempferol: ANOVA $P = 0.02$; the ANOVA was conducted although the homogeneity of variance was somewhat disturbed for kaempferol (Levene's test, $P = 0.01$). Quercetin: ANOVA $P = 0.02$. Kaempferol and quercetin concentrations did not differ between C_{EF} and C_{I-EF} leaves; n = 9–10 biological replicates.

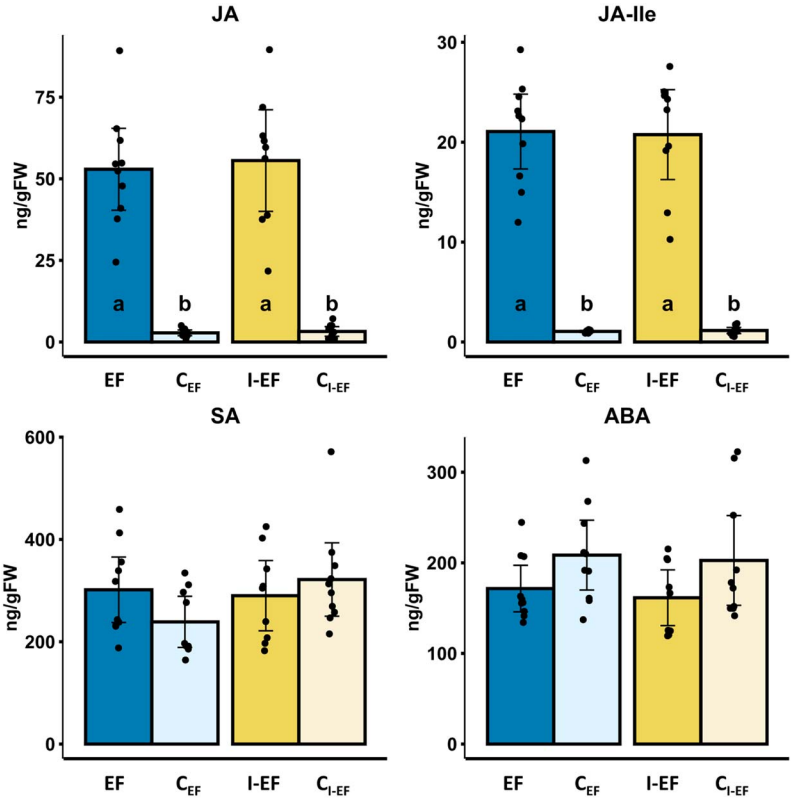

Figure 5. Impact of *X. luteola* infestation on phytohormone concentrations in *U. minor* leaves. The concentrations of jasmonic acid (JA), jasmonic acid–isoleucine (JA-Ile), salicylic acid (SA) and abscisic acid (ABA) were measured in EF (leaves with eggs and 1 day of larval feeding upon trees with no prior infestation) and in I-EF (leaves with eggs and 1 day of larval feeding upon trees that had been exposed to a *X. luteola* infestation in the previous growing season), as well as in untreated control leaves located below EF and I-EF leaves on the same trees (C_{EF} and C_{I-EF}). Concentrations of phytohormones were compared using Welch ANOVA and, in the cases of a significant result ($P < 0.05$), the Games–Howell test was applied (different letters indicate significant differences at $P < 0.05$). Bars represent mean ± 95% CI of n = 9–10 biological replicates.

However, when comparing the egg depositions on EF and I-EF trees per 3-week interval, previously infested I-EF elm trees tended to receive fewer eggs within the first 3 weeks of the oviposition period (Wilcoxon signed-rank test, $P = 0.078$; Figure 3) than EF trees (mean ± SE of calculated # eggs on EF tree: 299 ± 138; I-EF tree: 97 ± 40). This difference levelled out the longer the oviposition period lasted.

Prior infestation of elm trees affects feeding-induced changes in flavonoid concentrations

Kaempferol concentrations in previously infested I-EF leaves were significantly higher (~1.7-fold) after egg treatment and 1 day of larval feeding than in undamaged C_{EF} and $C_{I\text{-}EF}$ leaves. Similarly, quercetin concentrations in I-EF leaves were significantly higher (~1.3-fold) after egg treatment and larval feeding than in C_{EF} and $C_{I\text{-}EF}$ leaves (Figure 4). In contrast, the concentrations of kaempferol and quercetin were only slightly, not significantly, higher in EF leaves after egg treatment and a brief, 1-day feeding period by neonate larvae compared with the concentrations in undamaged C_{EF} leaves of the same tree (~1.2-fold change in EF vs C_{EF}). Kaempferol and quercetin concentrations did not differ between C_{EF} and $C_{I\text{-}EF}$ leaves.

Phytohormone concentrations in leaves from EF trees and previously infested I-EF trees do not differ after a brief larval feeding period

After 1 day of feeding by neonate larvae, the concentrations of JA and JA-Ile were significantly higher in the locally damaged leaves of EF and I-EF trees than in untreated control leaves downstream (C_{EF} and $C_{I\text{-}EF}$) (Figure 5). However, the concentrations of JA and JA-Ile were induced to a similar extent in the damaged leaves of both EF and I-EF trees. Thus, the induction of JA signalling by the most recent feeding damage was not affected by the prior infestation of the I-EF trees. The concentrations of SA did not differ significantly between the damaged and undamaged leaves of previously infested trees and those without a prior infestation (Figure 5). Abscisic acid concentrations tended to decrease by ~20% in response to feeding damage. However, the concentrations of ABA in the most recently feeding-damaged leaves from EF and I-EF trees did not differ significantly from those in undamaged C_{EF} and $C_{I\text{-}EF}$ leaves (Figure 5). Taken together, the prior infestation of the I-EF trees did not affect the phytohormone concentrations of the most recently infested trees.

Previously infested I-EF trees and EF trees show similar transcriptional responses in their stress-related genes and epigenetic marker genes to the most recent larval feeding damage

The expression of two genes involved in flavonoid biosynthesis—*UmPAL* and *UmANS*—was significantly induced in the leaves of both EF and I-EF trees responding to a 1-day feeding period by neonate larvae. Likewise, the expression of the

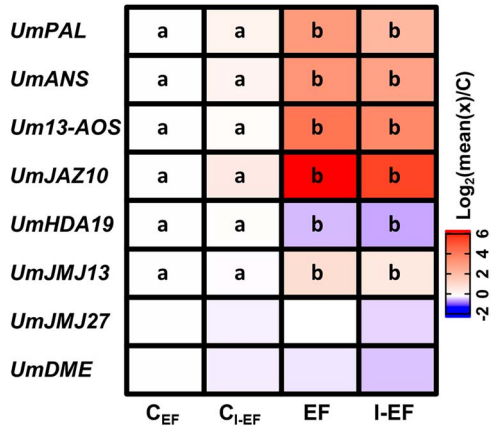

Figure 6. Expression of *U. minor* genes involved in phenylpropanoid biosynthesis, JA biosynthesis and regulation and histone and DNA modification. Transcript abundance was measured in EF (leaves with *X. luteola* eggs and 1 day of larval feeding upon trees with no prior infestation), I-EF (leaves with eggs and 1 day of larval feeding upon trees that had been exposed to a *X. luteola* infestation in the previous growing season) and untreated control leaves located below the treated leaves (C_{EF} and $C_{I\text{-}EF}$). Transcript abundance is expressed as the $\log_2$-fold change relative to C_{EF}. *UmPAL*: phenylalanine ammonia lyase; *UmANS*: leucoanthocyanidin dioxygenase; *Um13-AOS*: 13-allene oxide synthase; *UmJAZ10*: jasmonate ZIM domain protein 10; *UmHDA19*: histone deacetylase 19; *UmJMJ13*: JUMONJI 13; *UmJMJ27*: JUMONJI 27 (histone demethylases); *UmDME*: transcriptional activator DEMETER (a DNA demethylase). We used the Kruskal–Wallis test and, in the cases of a significant result ($P < 0.05$), the Wilcoxon signed-rank test as a post hoc test. Different letters indicate a significance at $P < 0.05$, $n = 10$ biological replicates.

JA biosynthesis gene *Um13-AOS* and the JA regulation gene *UmJAZ10* was induced in leaves being fed upon from EF and I-EF trees when compared with untreated C_{EF} and $C_{I\text{-}EF}$ leaves (Figure 6). However, a prior ELB infestation did not alter the expression intensity of these genes.

We also analysed the expression of genes involved in histone and DNA modifications. Larval feeding significantly reduced the expression of the histone deacetylase *UmHDA19* in both EF and I-EF leaves when compared with C_{EF} and $C_{I\text{-}EF}$ leaves. The expression of the histone lysine demethylase *UmJMJ13* was moderately but significantly induced by larval feeding upon EF and I-EF leaves when compared with C_{EF} and $C_{I\text{-}EF}$ leaves. The expression of another histone lysine demethylase, *UmJMJ27*, and of the DNA demethylase *UmDME* was slightly, but not significantly, reduced in response to the most recent damage to EF and I-EF leaves (Figure 6). Again, none of these feeding-induced changes in expression appeared to be affected by a prior infestation.

Discussion

Our study shows that infestation of elm by ELB egg deposition and feeding damage exerts moderately positive, long-term effects on elm defences against a further ELB infestation in leaves regrown after a simulated winter. We found that ELB

development and performance were impaired on previously infested trees. Thus, a prior infestation can benefit the tree in the subsequent growing season, in addition to the short-term benefit that ELB egg deposition can confer on elm defences against hatching larvae (Austel et al. 2016). Our results show that the detrimental effects of a prior infestation on ELB performance in the subsequent growing season are associated with long-term effects on feeding-induced flavonoid concentrations, which were found to be higher in previously infested I-EF leaves than in EF leaves. However, this difference was not reflected in the levels of phytohormones (JA, JA-Ile, SA and ABA), or in the expression of genes involved in flavonoid biosynthesis and epigenetic modifications. In the following, we will discuss our data with respect to the questions of how the elms' transcriptional, phytohormonal and metabolite responses to an ELB infestation were affected by a prior infestation, how they might be related to each other and how the changes induced by the prior infestation might have contributed to the ecological, long-term effect on ELB performance that we observed.

The role of elm epigenetic marks and phytohormones in regulating the long-term effects of ELB infestation

We detected two genes involved in histone modifications that were regulated by a current ELB infestation: the histone deacetylase *UmHDA19* was downregulated, and the histone demethylase *UmJMJ13* was weakly induced. However, the differential regulation of these genes was not affected by a prior infestation, neither in locally treated leaves nor in 'systemic' leaves, i.e. undamaged control leaves, of EF and I-EF trees (see Figure 6 and Table S7 available as Supplementary data at *Tree Physiology* Online).

Along with JAZ10 and several other proteins, HDA19 is known to form a complex that regulates the transcription of JA-responsive genes in *Arabidopsis* (Zhou et al. 2005, Wasternack and Hause 2013, Singh et al. 2016, Jiang et al. 2020). In ELB-infested elm, *UmJAZ10* was upregulated in the infested leaves of I-EF and EF trees. The transcriptional changes were accompanied by enhanced levels of JA in the leaves. In *A. thaliana*, JA induces the degradation of JAZ, thus activating the transcription of JA-responsive genes Wasternack C and Song S (2017); however, JA also induces the expression of *JAZ10,* thus forming a feedback loop and regulating (dampening) the JA response again (Chung et al. 2008). It remains an open question what the precise role of HDA19, in concert with JAZ10, is in the feeding-induced response of elm. The upregulation of *UmJMJ13* might be connected to the activation of stress resistance genes as in wounded *A. thaliana* (Ikeuchi et al. 2017). Here, our data clearly show that ELB infestation changes the expression of elm genes involved in shaping the chromatin status.

Besides histone modifications, biotic stresses can increase genome-wide DNA methylation and reduce the methylation of stress-induced genes, thereby shifting transcription in favour of stress-responsive genes (Peng and Zhang 2009, Thiebaut et al. 2019). We did not find significant changes in the expression of the DNA demethylase *UmDME*. However, since a plant can have several demethylases, this does not mean that demethylation processes do not play a role in elm resistance against herbivory.

With respect to genes involved in phytohormone biosynthesis and signalling, no differences were found between I-EF and EF leaves in the expression levels *Um13-AOS* (JA biosynthesis) and *UmJAZ10* (JA-induced signal transduction). This result was reflected by similar concentrations of JA and JA-Ile in I-EF and EF leaves. The application of MeJA is known to improve plant defences against subsequent herbivory occurring 2–5 weeks later (Mageroy et al. 2019, Chen et al. 2021). Here, we can only speculate as to whether JA very likely being induced during the first infestation by ELB also caused changes in elm trees that later impaired ELB performance.

We did not find significantly enhanced SA concentrations in elm leaves upon egg deposition and feeding, whereas our previous study detected enhanced SA concentrations in elm leaves that had first received ELB eggs and were later damaged by the feeding of hatching ELB larvae (Schott et al. 2022). However, this might be due to differences between elms of different origin, e.g. seed-grown elms from a tree nursery in our previous study and clonal elms in the present study (Zeier 2005, Kaurilind and Brosché 2017).

The impact of elm metabolites on the ecological effects of prior ELB infestation

Our finding of ELB performing worse on trees that had been exposed to an infestation in the previous year is in line with earlier studies of the performance of herbivores on deciduous trees that had been infested a year, or several years, earlier by feeding insects but without egg depositions on leaves (Valentine et al. 1983, Neuvonen et al. 1987, Ruuhola et al. 2007). However, a prior infestation has not always been found to improve anti-herbivore defences in the next growing season (e.g. Carroll and Quiring 1993, Osier and Lindroth 2004), suggesting that the ecological effect of a previous infestation may depend on several factors, such as the seasonal timing of the infestation (Chen et al. 2021), the tree species (Neuvonen et al. 1987), the feeding strategies of the herbivore (Nykänen and Koricheva 2004) and/or the availability of nutrients (Osier and Lindroth 2004).

While ELB egg-laying is known to lead to elm responses that reduce the performance of hatching ELB larvae (Austel et al. 2016), our study here shows that a pre-infestation can actually enhance this egg-mediated short-term effect on the defence of the currently infested tree. It remains an open question whether a previous infestation would also have an effect on the defence of the currently infested tree if no egg treatment had taken place and the short-term egg-mediated enhancer effect was missing.

Further research including elms treated with an initial infestation and—in a following season—with a second infestation that excludes egg deposition could help to elucidate the impact of egg depositions on the priming effect of pre-infestation. It would also be interesting to explore whether egg-induced indirect defences, i.e. the attraction of ELB egg parasitoids to egg-laden elm (Meiners and Hilker 2000), can be primed by an infestation of elms in the previous growing season.

In our study, the slightly enhanced levels of the phenylpropanoids quercetin and kaempferol in previously infested elm leaves might have contributed to the moderately poorer performance of ELB on these trees. We suggest this based on our earlier studies showing that (i) ELB larval mortality was higher on elm leaves treated with high levels of a kaempferol glycoside (robinin) than on untreated leaves (Austel et al. 2016) and (ii) ELB larvae feeding on previously egg-laden leaves suffered higher mortality than those feeding on egg-free leaves, which contained lower levels of quercetin than the egg-laden, feeding-damaged leaves (Austel et al. 2016, Schott et al. 2022). Several studies of interactions between other plant and insect species have shown that phenylpropanoids, including flavonoids like quercetin and kaempferol, impair insect performance (Simmonds 2001, 2003). Furthermore, the levels of elm flavonoids have also been shown to significantly increase in response to infection of an *U. minor* genotype by *Ophiostoma novo-ulmi*, the fungus causing Dutch elm disease (DED). Although this genotype is a DED-susceptible elm line, the pathogen-induced levels of flavonoids reached the levels present in DED-resistant elm lines. These fungus-induced high flavonoid levels might be considered an effort to at least limit fungal growth within the tree (Sobrino-Plata et al. 2022). The concentrations of phenylpropanoid derivatives are well known to increase in other tree species in response to phytopathogens (e.g. Miranda et al. 2007, Ullah et al. 2017) and herbivory (e.g. Lattanzio et al. 2006). Still, we cannot exclude the possibility that in our study, the concentrations of other secondary and primary elm metabolites, as well as the physical leaf structures, might have been affected by the prior ELB infestation and contributed to the impaired performance of ELB on re-infested trees.

Short-term priming by ELB egg deposition leads to enhanced *PAL* expression after a 24-h larval feeding period. The enhanced *PAL* expression in previously egg-laden, feeding-damaged leaves is associated with higher concentrations of quercetin and kaempferol (Schott et al. 2022). However, the higher concentrations of phenylpropanoids we found in I-EF compared with EF leaves were not reflected by higher levels of *UmPAL* transcripts in I-EF leaves. If a prior infestation leads to an earlier or a faster induction of defence-related genes in the event of a subsequent infestation (Hilker et al. 2016), potential differences in *UmPAL* expression between naïve and previously infested elms might have already levelled out after 24 h of feeding.

In addition to the effects of a prior ELB infestation on the insects' developmental time and weight, we observed more delayed egg depositions on previously infested trees. Elm leaf beetle females on I-EF trees had a lighter initial adult weight and therefore might need to feed for longer until their eggs are sufficiently mature for deposition. Alternatively, previously infested trees might be less susceptible to oviposition; in our experiment, eggs were eventually laid on I-EF trees because the ELB females were offered no other host plants. The studies of the alder leaf beetle (*Agelastica alni*) and the herbivorous sawfly *Nematus oligospilus* have shown that these species avoid oviposition on previously infested or mechanically defoliated trees (Dolch and Tscharntke 2000, Valladares et al. 2020).

What are the ecological consequences of reduced ELB performance on elm trees? Herbivory on trees can cause reduced seed set and lower quality seed production, as shown for pinyon pines (*Pinus edulis*) and holm oak (*Quercus ilex*) (Mueller et al. 2005, Canelo et al. 2018), and negatively impact the regeneration of trees, especially under challenging conditions such as drought (Canelo et al. 2018). Shestakov et al. (2020) studied the impact of recurring herbivory on the growth of birch (*Betula pubescens*), poplar (*Populus tremula*), spruce (*Picea abies*) and pine (*Pinus sylvestris*); they demonstrated that even a small, but recurring, decrease in insect herbivory on deciduous and coniferous forest trees over a period of four seasons had a pronounced positive impact on tree growth and almost doubled biomass production. Based on our data, we suggest that the pre-infestation effect on ELB performance in the following season can limit ELB population dynamics over time and thus limit tree damage. This effect may even increase when the ELB succeeds in developing several generations per season (Dreistadt 2004, Rodrigo et al. 2019). The effects of a prior infestation on elm defences against ELB may work in tandem with egg-mediated, short-term effects and help to limit ELB population growth, thereby reducing the herbivory pressure on elm trees. We suggest that even a minor reduction of ELB herbivory by pre-infestation-mediated long-term priming and egg-mediated short-term priming may help to limit the tree's fitness losses (seed production, biomass production and competitiveness) due to ELB infestation.

Concluding remarks

The long-term response of elm to a prior infestation adds a further dimension to our understanding of how elm trees prepare themselves for defence against impending herbivory.

Increased concentrations of the phenylpropanoids kaempferol and quercetin are not only linked with the positive, short-term effect of insect egg deposition on elm defences against ELB larvae (Austel et al. 2016, Schott et al. 2022) but also were found to be associated with the long-term benefit of prior infestation for anti-herbivore defences. However, in contrast

to the egg-mediated, short-term priming of elm defences, the long-term effect was not linked with an enhanced expression of *PAL* in previously infested trees. Future studies of the activities of enzymes involved in the phenylpropanoid pathway should shed light on whether the concentration of phenylpropanoids is regulated at a level other than the transcriptional one.

A current ELB infestation was shown to differentially regulate epigenetic marker genes, while the expression levels of infestation-induced genes did not appear to be affected by a previous infestation. Further studies are needed to evaluate whether the epigenetic marks of defence-related genes persist after an infestation and contribute to long-term stress 'memory' in elm, in the sense of epigenetic memory marks, as defined by Avramova (2015). A comparative analysis of the DNA methylation profiles of leaf buds that may have long-term epigenetic memory, rather than of infested leaves that are shed in autumn, could further elucidate how elm trees store information about previous infestations (Le Gac et al. 2018).

Beyond that, further research could search for the elicitors of elm defences against ELB. So far, several compounds associated with insect eggs are known to induce plant defences against the eggs (Hilker and Fatouros 2015, Hundacker et al. 2022), whereas a wide range of other chemicals released by insect larvae have also been identified as the elicitors of plant defences against the feeding larvae (Jones et al. 2022, Snoeck et al. 2022). Insect egg-associated elicitors of primed plant responses to feeding larvae are known to be present in secretions associated with eggs, i.e. in the oviduct secretion of the ELB (Austel et al. 2016) and in female accessory reproductive gland secretion in a butterfly (Paniagua Voirol et al. 2020). However, the chemical nature and structure of the priming-relevant compound(s) associated with ELB eggs and oviduct secretion have yet to be identified.

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Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Conflict of interest

None declared.

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Data availability

All relevant data are within the paper and its Supporting Information files.

Authors' contributions

J.S. and M.H. conceptualised, designed and organised the study. J.S. performed the experiments and evaluated the data. F.J. supported the study, especially with respect to the qPCR analyses. J.S. wrote a first draft of the manuscript, and all authors contributed to, and agreed upon, the final version.

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