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Research

Small-scale indirect plant responses to insect herbivory could have major impacts on canopy photosynthesis and isoprene emission

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Summary

- Insect herbivores cause substantial changes in the leaves they attack, but their effects on the ecophysiology of neighbouring, nondamaged leaves have never been quantified in natural canopies. We studied how winter moth (*Operophtera brumata*), a common herbivore in temperate forests, affects the photosynthetic and isoprene emission rates of its host plant, the pedunculate oak (*Quercus robur*).
- Through a manipulative experiment, we measured leaves on shoots damaged by caterpillars or mechanically by cutting, or left completely intact. To quantify the effects at the canopy scale, we surveyed the extent and patterns of leaf area loss in the canopy.
- Herbivory reduced photosynthesis both in damaged leaves and in their intact neighbours. Isoprene emission rates significantly increased after mechanical leaf damage. When scaled up to canopy-level, herbivory reduced photosynthesis by 48 ± 10%.
- The indirect effects of herbivory on photosynthesis in undamaged leaves (40%) were much more important than the direct effects of leaf area loss (6%). If widespread across other plant–herbivore systems, these findings suggest that insect herbivory has major and previously underappreciated influences in modifying ecosystem carbon cycling, with potential effects on atmospheric chemistry.

Introduction

Interactions between plants and insect herbivores are among the most common ecological interactions (Strong *et al.*, 1984; Schoonhoven *et al.*, 2005). By influencing plant distribution, abundance and evolution, insect herbivores can have major impacts on community composition, primary productivity and biosphere–atmosphere interactions (Belovsky & Slade, 2000; Karl *et al.*, 2008; Metcalfe *et al.*, 2014).

By removing plant tissue (*a direct effect* of herbivory), insect herbivores can substantially reduce photosynthesis. The loss of tissue often changes both primary (basic metabolic processes like respiration) and secondary (e.g. production of defensive chemicals) plant metabolism (Herms & Mattson, 1992; Kerchev *et al.*, 2012). This can lead to changes in the nutrient content or toxicity of the plant. Plants can respond to herbivory also by emitting volatile organic compounds (VOCs; Rowen & Kaplan, 2016). These changes, often triggered as defensive reactions, can spread to systemic undamaged tissue and affect all parts of the plant (Agrawal, 2000; Staudt & Lhoutellier, 2007; Wu & Baldwin, 2009).

Insect-induced changes in chemistry and metabolism can further alter the photosynthetic capacity of the remaining leaf tissue (*an indirect effect* of herbivory, Zangerl *et al.*, 2002; Nykänen & Koricheva, 2004; Nabity *et al.*, 2009). Leaf damage often triggers upregulation of defence-related genes and downregulation of genes related to photosynthesis (Bilgin *et al.*, 2010). Nevertheless, previous studies have found both increased ('compensatory photosynthesis') and decreased photosynthetic rate as a response to herbivory (Zangerl *et al.*, 2002; Nykänen & Koricheva, 2004; Nabity *et al.*, 2009). Similarly, VOC emission can either increase (as defensive reaction through plant–predator communication or plant–plant signalling) or decrease after leaf damage (Loreto & Sharkey, 1993; Dicke & Baldwin, 2010; Rowen & Kaplan, 2016). The exact plant response to herbivory depends on the characteristics of the specific species interaction, for example on the diet breadth (e.g. specialist vs generalist) or feeding guild (e.g. chewing vs sap-sucking) of the herbivore (Nykänen & Koricheva, 2004; Kessler & Halitschke, 2007; Rowen & Kaplan, 2016).

Isoprene is one of the most abundant plant-emitted hydrocarbons (Guenther *et al.*, 1995; Wang & Shallcross, 2000), produced by many long-lived woody species (Dani *et al.*, 2014). It is

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often emitted in small quantities alongside photosynthesis (Rasulov *et al.*, 2009), but also plays a key role as a stress chemical helping the plant to cope with high temperature (Sharkey & Singsaas, 1995; Rasulov *et al.*, 2010). Because isoprene influences the formation and lifetime of lower tropospheric pollutants (Fehsenfeld *et al.*, 1992; Fuentes *et al.*, 2000), changes in isoprene emissions can influence atmospheric chemistry (Mentel *et al.*, 2013; Kravitz *et al.*, 2016). For estimating the effects of insect herbivory on atmospheric chemistry, quantifying herbivory-induced changes in isoprene emissions is of key interest.

To date, most studies assessing the link between herbivory and photosynthesis or isoprene emission have used cultivated model plant species (mostly species in the Brassicaceae or Solanaceae), simulated herbivory (Portillo-Estrada *et al.*, 2015), or controlled glasshouse environments (Kessler & Halitschke, 2007). The effect of herbivory (including its *indirect effects)* on photosynthesis or isoprene emissions in natural systems thus remains largely unknown. In addition, these effects have often been studied at the scale of individual plants or plant parts, and remain poorly quantified at larger scales. This prevents us from drawing conclusions about the large-scale influence of insect herbivory on carbon (C) cycling and atmospheric chemistry.

Using a manipulative experiment, we investigated how a common insect herbivore affects photosynthesis and isoprene emission rate of its host plant in a natural broadleaf deciduous forest. As a study system, we used the pedunculate oak (*Quercus robur*) and caterpillars of the winter moth (*Operophtera brumata*), both of which are common species throughout temperate woodlands. We measured rates of photosynthesis and isoprene emissions in intact leaves, leaves eaten by herbivores, intact leaves close to eaten leaves (to quantify the systemic effects), and leaves subject to mechanical damage (to gain insights into how the potential herbivory-induced responses are triggered). Specifically, we addressed the following questions: (1) do photosynthetic and/or isoprene emission rates of oak leaves change following leaf damage? (2) Is the effect different between herbivore-induced damage vs mechanical wounding? (3) Are damage-induced responses restricted to damaged leaves, or can changes in photosynthetic and/or isoprene emission rates be observed on intact leaves close to their damaged neighbour? (4) What are the total effects of herbivory-induced leaf area loss (*direct effect*) and changes in the remaining leaf tissue (*indirect effect*) at the canopy scale?

Materials and Methods

Experimental setup

The study was carried out during the springs and summers of 2015–2016 on 10 oak trees (*Quercus robur* L.) in Oxfordshire, UK. Five of the oaks were mature trees (mean diameter at breast height (dbh) 67.2 ± 5.4 cm SEM) located in Wytham Woods (51°46′27.48″N, 1°20′16.44″W, 160 m asl), and the remaining five were young (mean dbh 13.6 ± 1.8 cm SEM) planted oaks by the John Krebs field station in Wytham (51°47′1.32″N, 1°19′1.2″W, 63 m asl). Oak is a strong isoprene emitter (Lehning *et al.*, 1999). On both sites, the oaks are naturally infested by caterpillars of the winter moth, which is a common generalist early-spring herbivore. The caterpillars emerge in synchrony with the budburst, and feed on the newly flushed leaves until June (Hunter, 1992). Relatively few herbivore species feed on the mature oak leaves later in the season (Feeny, 1970) Oaks in our study area do not reach their full photosynthetic capacity until late June, (Morecroft *et al.*, 2003), creating a time lag between the peak herbivory and the peak photosynthesis. For herbivores to have substantial impact on photosynthesis in this system, their effect should carry over until the oak has reached its full photosynthetic capacity.

Between 11 and 15 May 2015 and 9 and 11 May 2016, when most leaves were still newly flushed, we identified 15 shoots (of *c.* eight leaves) with only intact leaves from each study tree and enclosed each shoot in a small mesh fabric bag (Supporting Information Fig. S1). We randomly assigned each bag into one of the three treatments: (1) *herbivore addition*, (2) *mechanical damage*, or (3) *control*, so that each tree had five bags of each treatment. For each of the *herbivore addition* bags we added a locally collected winter moth caterpillar, and let it feed on the leaves for 3–5 d until at least two of the leaves showed signs of feeding damage. Because the effect of damage often depends on its type and amount (Wu & Baldwin, 2009; Portillo-Estrada *et al.*, 2015), each *herbivory addition* shoot was paired with a *mechanical damage* shoot immediately after the caterpillars had been removed from the mesh bags. The damage on the herbivory shoots was then replicated by tearing or punching holes with a cork borer in the leaves in the mechanical damage treatment (Fig. S2). *Control* shoots were left intact. The timing of the manipulations coincided with the peak herbivory in the area (Charmantier *et al.*, 2008). The mesh bags were left around the shoots to prevent additional herbivory until 25 June 2015 or 28 June 2016, when the amount of insect herbivory had levelled off.

One month after the application of the treatments, we randomly chose three shoots from each tree (one *herbivory addition* shoot, one *mechanical damage* shoot, and one *control* shoot) for gas exchange measurements. The few control shoots ($n = 6$) that showed signs of damage were excluded from further measurements. From each *herbivory addition* and *mechanical damage* shoot we measured two leaves: one damaged and one intact. From each *control* shoot we measured one intact leaf. This setup allowed us to measure five leaf-level treatments: damaged leaf in herbivory treatment, undamaged leaf in herbivory treatment, damaged leaf in mechanical treatment, undamaged leaf in mechanical treatment and intact control leaf. We constructed photosynthetic light response curves (over the period of 28 July–25 August 2015) for 49 leaves from 10 trees and photosynthesis-CO_2 (A/C_i) -curves (over the periods of 26 August–10 September 2015, and 11 July–11 August 2016) for 79 leaves from 10 different trees (six of the trees were measured on both years) belonging to all of the five leaf-level treatments The timing of the gas exchange measurements corresponded to the peak photosynthetic activity of oak in the study area (Morecroft *et al.*, 2003).

On each leaf, we measured an intact part of an area of 2.5 cm^2 of the leaf with an infrared gas analyser (CIRAS-2, PP-Systems, Hitchin, UK). For the light response curves, we took five point

measurements on 15 different light intensities between 2000 and 0 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR). For the A/C_i curves, we measured the photosynthetic rate under 10 different CO_2 concentrations between 1300 and 30 ppm. All of the raw photosynthesis measurements were processed using the protocol provided by PP-Systems (ppsystems.com) for the CIRAS-2 to apply corrections for the measured variables. The resultant variable used in the analyses was photosynthetic rate per unit leaf area, expressed as μmol CO_2 m^{-2} s^{-1}. See Methods S1 for details on the gas exchange measurements.

In order to study how herbivory and leaf damage affect the production of isoprene by the oak, we measured isoprene emission rate of 32 leaves from seven trees, using the same leaves (and thus the same five leaf-level treatments) as for the A/C_i curves with a portable gas chromatograph (iDirac; Methods S2), 21 July–9 August 2016. iDirac is a novel gas chromatograph, designed for *in-situ* use. Here we report its use for the first time in a field study. We attached the iDirac directly into the CIRAS-2 system to allow for simultaneous measurements of isoprene production and photosynthetic rate.

After measurements were taken, the leaves were photographed to estimate the leaf area lost to herbivory. To estimate the natural level of insect herbivory on the study trees throughout the growing season, we collected 15 additional shoots from each tree on four time points (16–28 May, 25 June, 14 July–10 August and 18 August 2015), and pressed and scanned the leaves. The area lost to herbivory of the photographed and scanned leaves were estimated as the percentage of missing area from the side of the leaf, from the tip, or as holes, using ImageJ software (NIH, MD, USA).

Extracting response parameters

In order to calculate the light-saturated photosynthesis, we fitted a Michaelis–Menten equation to the light response data for each leaf separately to estimate the parameters for the maximum light-saturated photosynthetic rate (A_{sat}) and the light intensity at which the gross photosynthetic rate is half of its maximum, K (Marino *et al.*, 2010). To obtain a measure of the mean dark respiration (R_d) for each leaf, we calculated the average photosynthetic rate on the light response curves when the light intensity was zero. To analyse the photosynthetic response to experimental treatments under different CO_2 concentrations, we constructed A/C_i response curves, where the photosynthetic rate (A) is modelled against the intercellular CO_2 mole fraction (C_i) (Farquhar *et al.*, 1980; Sharkey *et al.*, 2007), allowing us to estimate three important photosynthetic parameters: maximum carboxylation rate, describing the activity of Rubisco (V_{cmax}); rate of photosynthetic electron transport (J_{max}); and triose phosphate use efficiency (TPU).

After fitting, all of the parameters were normalized to 25°C (Harley *et al.*, 1992; Sharkey *et al.*, 2007) to reduce variation caused by different ambient temperatures. For most leaves ($n = 65$) the Farquhar *et al.* (1980) model could be fitted to the data. For some leaves ($n = 14$) the model failed to estimate at least one of the parameters. These leaves were omitted from the further analyses of the treatment effects on A/C_i parameters. To study possible changes in leaf conductance, we extracted the mean stomatal conductance (g_s) recorded by the gas analyser during the A/C_i curve measurements. From those leaves of which only light response was measured (24 leaves), we used mean stomatal conductance of the light response curve. Single outlier values of stomatal conductance, K and isoprene emission were removed from further analyses. See Fig. 2 for final sample sizes per parameter.

In order to estimate isoprene emissions, the height of each isoprene peak in the gas chromatogram was measured and converted into mixing ratios (ppb) by using calibration measurements with known isoprene concentrations. The mixing ratios were scaled with the known air volume, area of leaf measured and flow rate to yield emission rates as nmol m^{-2} s^{-1}. Because isoprene emission is strongly influenced by temperature, we corrected the measured emission values for temperature (Guenther *et al.*, 1993, 1995), yielding the standard emission factor of isoprene (as μg m^{-2} h^{-1}), I_S (in 303 K and 1000 μmol m^{-2} s^{-1} of photosynthetically active radiation). See Methods S1 for details on the model fitting and the temperature corrections.

In order to describe the photosynthetic rate of the study leaves in natural conditions, we extracted values from the light-response and A/C_i curves for photosynthetic rates at ambient CO_2 concentration (400 ppm) and in light intensity that corresponds to typical full light conditions (1000 μmol m^{-2} s^{-1} of photosynthetically active radiation). This parameter (A_{1000}), was used to assess the correlation between photosynthesis and isoprene emission rate, and to scale up the effects of herbivory from leaf scale to the canopy level.

Statistical analyses

In order to test for effects of our experimental treatments on photosynthesis and isoprene emission, we built a separate linear mixed effects model for each of the key response parameters described above. Each photosynthesis-related response parameter (A_{sat}, K, R_d, V_{cmax}, J_{max}, TPU, g_s) was modelled as a function of leaf-level treatment (a categorical variable with five levels), site (Wytham Woods or John Krebs field station), mean leaf temperature (to account for any remaining variation by the ambient temperatures), year (2015 or 2016, for the parameters that had been measured in both years), and the percentage of leaf damage as explanatory variables. Time of the day was assumed to have a nonlinear effect, and was added as general additive smoother. To avoid spurious treatment effects due to small sample sizes, interactions were not included (Zuur, 2009). Tree identity and shoot identity (nested within tree identity), were included as random factors (random intercepts) to account for nonindependence of leaves on the same shoots and trees. The same approach was used to model I_S, except that variance structure was allowed to vary between the leaf treatments to allow for unequal variances across these groups. For each response variable, the full model was simplified by dropping one explanatory variable at a time. The change in the model fit was assessed using likelihood ratio tests. Fixed factors that did not improve model fit were dropped from

the final model (Crawley, 2007). Where leaf type was significant, a *post-hoc* Tukey's honest significant difference (HSD) test was applied to assess which of the five leaf treatments differed significantly from one other. Because of the adjusted variance structure in the isoprene model, the pairwise leaf treatment comparisons were carried out estimating least square means.

In order to analyse the relationship between isoprene emission and the photosynthetic parameters measured simultaneously (A_{1000}, V_{cmax}, J_{max} and TPU), we built linear, exponential and quadratic models in which the isoprene emission rate was modelled as a function of each selected photosynthetic parameter. We then estimated the model fit by comparing the adjusted r^2-values between the different models (linear, exponential and quadratic), and selected the model with the highest r^2 value for each of the parameters.

In order to test for the differences in the amount of leaf damage between the two damage treatments (mechanical and herbivory) and naturally occurring damaged leaves, we built a linear model with proportion of damage as a function of damage type (herbivore addition, mechanical, natural). To test for patterns in natural herbivory levels, we built a linear model of proportion of damage as a function of the site and the collection date. Proportions were arcsine-square root-transformed in order not to violate model assumptions (Crawley, 2007). For all models, the model assumptions were tested by visually examining plots of residuals against fitted values for the homoscedasticity of residuals, and a Quantile-Quantile plot for the normal distribution of the residuals. All analyses were conducted using R v.3.4.1 (R Core Team, 2017) and the packages LME4 (Bates *et al.*, 2015), multcomp (Hothorn *et al.*, 2008), NLME (Pinheiro *et al.*, 2017), gamm4 (Wood & Scheipl, 2017) and LSMEANS (Lenth, 2016).

Quantifying the effects of herbivory on leaf and canopy scales

In order to estimate the effects of herbivory on photosynthesis and isoprene emission at the canopy scale, we combined three types of data: (1) the proportion of leaf area loss per leaf under natural conditions (direct effect), (2) the effect of insect herbivory on A_{sat} or I_S per unit leaf area (indirect effect), and (3) information on natural patterns of herbivory in the oak canopy. Control leaves, which were intact leaves on intact shoots were set as a reference point to describe photosynthesis and isoprene emission in the absence of herbivory. To estimate the leaf-scale effect of herbivory on the light-saturated photosynthesis or isoprene emission rate, we first multiplied the per leaf unit area rate of a leaf damaged by herbivores with the proportion of remaining leaf area in the corresponding leaf type, yielding a 'per leaf' - rate. We then compared this to a 'per leaf' - rate of an intact control leaf:

$$\text{light saturated leaf scale effect}_t = \frac{A_t \times (1 - D_t)}{A_{t=1}} - 1 \qquad \text{Eqn 1}$$

(A, light-saturated assimilation rate (A_{sat}) or the isoprene emission rate; D, proportion of leaf area loss per leaf type (= direct effect, between 0 and 1); t three different leaf types (1 = intact leaf in a completely intact shoot, 2 = intact leaf in an herbivory treatment, 3 = damaged leaf)). For the intact leaves in the herbivory treatment, the leaf-scale effect was simply the percentage change in the photosynthetic or isoprene emission rate, indicating a 'shoot-level effect' of herbivory spreading from the damaged leaves to the intact neighbours.

We estimated the effect of herbivory at the canopy level with two different methods. First, to estimate the herbivory effect at the level of the canopy for the maximum light-saturated photosynthesis and isoprene emission rate, we multiplied the light saturated leaf-scale effect of each leaf type by the proportion of the respective leaf type in the canopy, and then summed these values over the three leaf types:

$$\text{light saturated canopy effect} = \sum_{t=1}^{3} \text{leaf scale effect}_t \times l_t \qquad \text{Eqn 2}$$

(t, three different leaf types; l, proportion of leaf type t in the canopy). For photosynthesis, this model estimates the maximum potential photosynthesis in full light (as $\mu mol\ m^{-2}\ s^{-1}$ of leaf area), without considering light transmission through the canopy.

Second, because photosynthesis is strongly affected by the amount of available light, we estimated the effect of herbivory on canopy photosynthesis when the diffusion of light through the canopy is taken into account. To estimate this, we used the Big Leaf approach of The Joint UK Land Environment Simulator ('JULES', Clark *et al.*, 2011) to estimate canopy assimilation, combined with an estimate for canopy respiration (Mercado *et al.*, 2007). The reduction of direct light through the canopy was calculated by Beer's law (Monsi & Saeki, 1953). As a result, our model estimates instantaneous big-leaf approximated net CO_2 assimilation rate. Assimilation is reduced proportional to the transmission of light through the canopy, whereas leaf respiration increases as light decreases:

$$\text{NPC} = \int_0^{\text{LAI}} A_{sat} \times \left(\frac{\text{PAR}}{K + \text{PAR}}\right) \times \left(e^{-k \times \text{LAI}}\right) - \left(0.5 - 0.05 \times \log_e\left(\text{PAR} \times e^{-k \times \text{LAI}}\right)\right) \times R_d \qquad \text{Eqn 3}$$

(NPC, canopy net photosynthesis (as $\mu mol\ m^{-2}\ s^{-1}$ of ground area); A_{sat}, light-saturated photosynthetic rate; k, light extinction coefficient; LAI, canopy leaf area index; PAR, light intensity at the top of the canopy; and R_d, dark respiration rate estimated from the Michaelis–Menten equation (Methods S1, Eqn S1)). The coefficient k was set to 0.5 as a previously used estimate for broadleaf forests (Clark *et al.*, 2011), LAI was set to 7.8 as measured previously for this field site (Fenn *et al.*, 2015) and PAR was set to 1000 $\mu mol\ m^{-2}\ s^{-1}$ as a standard daytime light intensity at the top of the canopy. We estimated canopy net photosynthesis for each leaf type (i.e. canopy consisting of only that leaf type), multiplied the estimates with the proportion of the respective leaf type observed in the canopy, and then summed these

values over the three leaf types. This estimate was then compared to an estimate of a canopy with intact leaves only. Finally, we included the direct effect by subtracting the proportion of leaf area loss at canopy level:

$$\text{canopy effect at diffused light} = \left(\frac{\sum_{t=1}^{3} \text{NPC}_t \times l_t}{\text{NPC}_{t=1}} - \text{D}_c \right) - 1 \quad \text{Eqn 4}$$

(t, three different leaf types; l, proportion of leaf type t in the canopy; and D_c, proportion of leaf area loss (= direct effect) at the canopy scale).

Data availability

The primary data for this article are available at the Knowledge Network for Biocomplexity (https://doi.org/10.5063/F1ZK5DV2).

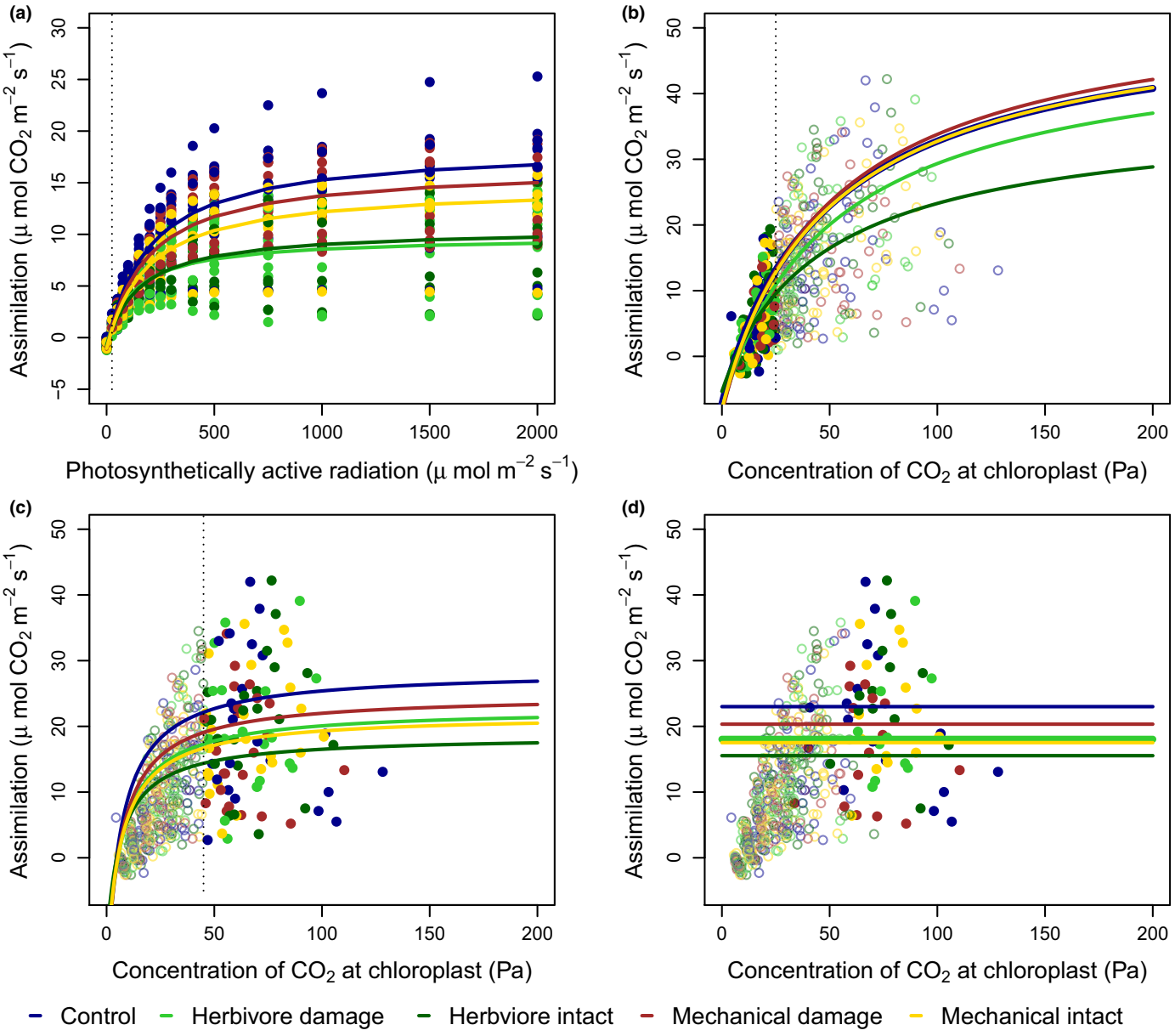

Fig. 1 The average model-predicted response curves of leaves of *Quercus robur* subject to herbivory by *Operophtera brumata*, mechanical damage or left as intact (control). (a) Photosynthetic response to light, (b) the maximum carboxylation rate (V_{cmax}), (c) the maximum electron transport rate (J_{max}) and (d) the maximum triose phosphate use efficiency (TPU). The original measurements are shown as points, and average model-fitted parameters per treatment are shown as lines. For (b–d), the solid points represent measurements used to estimate the corresponding parameter (i.e. when $[CO_2] < 25$ Pa for V_{cmax}, $[CO_2] > 45$ Pa for J_{max}, and assimilation at its maximum for TPU, see Supporting Information Methods S1 for details), and the circles show the remaining measurements. The dashed vertical lines at C_i concentrations of 25 (b) and 45 Pa (c) represent the points at which the limiting factor of the photosynthetic rate was assumed to change (from Rubisco limited into RuBP limited photosynthesis). The data represent measures from both field sites, and in (b–d) during both measuring years. Note that the effect of site and year has been taken into account in the statistical analyses. See Fig. S3 for the complete A/C_i response curves per treatment.

Results

Herbivory under natural and experimental settings

There was no difference between the natural levels of herbivory between the two sites ($t = -0.55$, df = 2, 1461, $P = 0.58$) and no change throughout the growing season ($t = -1.65$, df = 2, 1461, $P = 0.10$), indicating that early-season herbivory is the dominant type of insect herbivory in the study system. Almost all shoots surveyed for natural herbivory had at least one damaged leaf: of the 175 shoots surveyed, only three (1.7%) were completely intact.

The mesh bags successfully prevented herbivores from colonizing the experimental shoots (94 of 100 control shoots remained intact). The amount of leaf damage did not differ between the two damage treatments (10.88% ± 1.84% in mechanical and 14.13% ± 1.91% in herbivore addition, $t = -0.90$, df = 2, 1086, $P = 0.37$), but was higher in leaves with experimental herbivory compared to naturally occurring herbivory (8.45% ± 0.39%, $t = 3.04$, $P = 0.002$ for herbivore addition and $t = 1.72$, $P = 0.09$ for mechanically damaged). Most leaf damage occurred at sides and tips, and only a small portion as holes (Table S1).

Treatment effects on photosynthesis and isoprene emission

Leaf treatment significantly influenced A_{sat} ($\chi^2 = 17.31$, $P = 0.002$, df = 4, 8; Table S2; Figs 1a, 2a), V_{cmax} ($\chi^2 = 9.51$, $P = 0.05$, df = 4, 11; Table S2; Figs 1b, 2d, S3), J_{max} ($\chi^2 = 11.23$, $P = 0.02$, df = 4,10; Table S2; Figs 1c, 2e, S3), g_s ($\chi^2 = 10.48$, $P = 0.03$, df = 4,10; Table S2; Fig. 2g) and I_S (Lratio = 23.15, $P < 0.001$, df = 4,9; Table S2; Fig. 2h). Both damaged and undamaged leaves in the herbivore addition shoots experienced a significant reduction in their A_{sat} and J_{max} compared to control leaves ($z = -4.26$, $P < 0.001$ damaged leaves and $z = -4.26$, $P < 0.001$ undamaged leaves for A_{sat}, $z = -38.92$, $z = -2.84$, $P = 0.03$ damaged leaves and $z = -3.24$, $P = 0.01$ undamaged leaves for J_{max}). V_{cmax} was different between leaves damaged mechanically and intact leaves in the herbivory treatment, but the difference was only marginally significant (Tukey's HSD test, $z = 2.55$, $P = 0.08$). The g_s was different between control and the undamaged leaf in the herbivory treatment ($z = -2.73$, $P = 0.049$). The light intensity at which the gross photosynthetic rate is half of its maximum (K, Fig. 2b), R_d (Fig. 2c), and TPU (Figs 1d, 2f, S3), on the other hand, were not influenced by leaf treatment. Mean leaf temperature significantly increased V_{cmax} ($\chi^2 = 4.21$, $P = 0.04$, df = 1, 11), J_{max} ($\chi^2 = 9.98$, $P = 0.002$, df = 1, 10), TPU ($\chi^2 = 9.93$, $P = 0.002$, df = 1, 6), R_d ($\chi^2 = 8.11$, $P = 0.004$, df = 1, 5) and g_s ($\chi^2 = 5.34$, $P = 0.02$, df = 1, 10). V_{cmax}, J_{max}, TPU and g_s were significantly different between the two sites ($\chi^2 = 5.07$, $P = 0.02$, df = 1, 11 for V_{cmax}; $\chi^2 = 5.58$, $P = 0.02$, df = 1, 10 for J_{max}; $\chi^2 = 5.34$, $P = 0.02$, df = 1, 6 for TPU and $\chi^2 = 5.95$, $P = 0.01$, df = 1, 10 for g_s), and V_{cmax} differed between the 2 years ($\chi^2 = 8.82$, $P = 0.03$, df = 1, 11).

Leaves damaged mechanically had significantly higher isoprene emission rate compared to control leaves and undamaged leaves in the herbivory treatment ($t = -6.57$, $P < 0.007$ and $t = -7.16$, $P < 0.004$, respectively). The isoprene emission rate per unit leaf area decreased with increasing percentage of leaf damage (Lratio = 8.32, $P = 0.004$, df = 1, 9). Isoprene emission rate correlated positively and significantly with photosynthesis (Fig. S4).

The effects of herbivory on leaf and canopy scales

Leaf area loss (the *direct effect* of herbivory) per leaf was 8.5% ± 0.4%. The *indirect effect* of herbivory (i.e. the herbivory-induced change in photosynthesis in the remaining leaf tissue) accounted for a 45.5% ± 10.1% reduction in the leaf-scale A_{sat} (Table 1). Hence, the indirect effect of herbivory was several magnitudes larger than the direct effect of leaf area loss. Within the shoots that had herbivory damage, the reduction in photosynthesis was almost identical between damaged leaves and their undamaged neighbors. When the *direct* and *indirect effects* and the proportion of damaged and undamaged leaves in the canopy were combined, 45.6% ± 7.6% of the light-saturated photosynthesis and 47.9% ± 9.5% of the net photosynthesis under diffused light was lost to herbivores at the canopy-scale (Table 1). The first estimate represents a canopy consisting only of sun leaves at full light, (see Table S3 for estimates on canopy-scale effects of herbivory on photosynthesis at lower light intensity), whereas the second estimate represents a canopy where light is reduced with increasing leaf area index due to shading. Despite the different assumptions of these estimates, the proportional change in photosynthesis due to herbivory is effectively the same.

In contrast to the photosynthesis, isoprene emission rates increased in the damaged leaves by 85.4 ± 115.6% compared to the intact control leaves, although the small number of samples and the associated large error makes drawing conclusions difficult. The shoot-level effect, where shoot-level herbivory affects undamaged leaves within the same shoot, was small (29.8 ± 32.1%) for isoprene. At the canopy-scale, the total effect of herbivory corresponded to a 52.5 ± 82.6% increase in isoprene emissions, but with large variation (Table 1).

Discussion

In this study herbivory substantially reduced photosynthesis in damaged leaves and in their intact neighbours. Isoprene emission rates significantly increased after mechanical leaf damage. At the canopy scale, these results indicate that even a relatively moderate intensity of herbivory (6% of canopy leaf area), leads to a 48% reduction in the potential photosynthesis and a 53% increase in isoprene emission rate, although the effect on isoprene emission was not statistically significant at the canopy-scale. Below, we will discuss each of our findings in turn.

Why does the photosynthetic rate change following leaf damage?

Previous studies on the indirect effects of herbivory on photosynthesis have reported increases (Oleksyn *et al.*, 1998; Nykänen & Koricheva, 2004), decreases (Oleksyn *et al.*, 1998; Nabity *et al.*, 2009) and no changes (Peterson *et al.*, 2004) in the assimilation

rates after leaf damage. In this study, leaf damage by herbivores lowered the maximum light-saturated photosynthetic rate (A_{sat}), maximum carboxylation rate (V_{cmax}) and the maximum electron transport rate (J_{max}). As stomatal conductance (g_s) correlates with photosynthesis (Wong *et al.*, 1979; Gago *et al.*, 2016), its responses to the treatments were similar to that of photosynthesis. These effects were visible several months after the initial damage. It is unclear whether photosynthesis had remained low during the entire period, or whether the reduction became observable only late in the season. Other studies have reported delayed

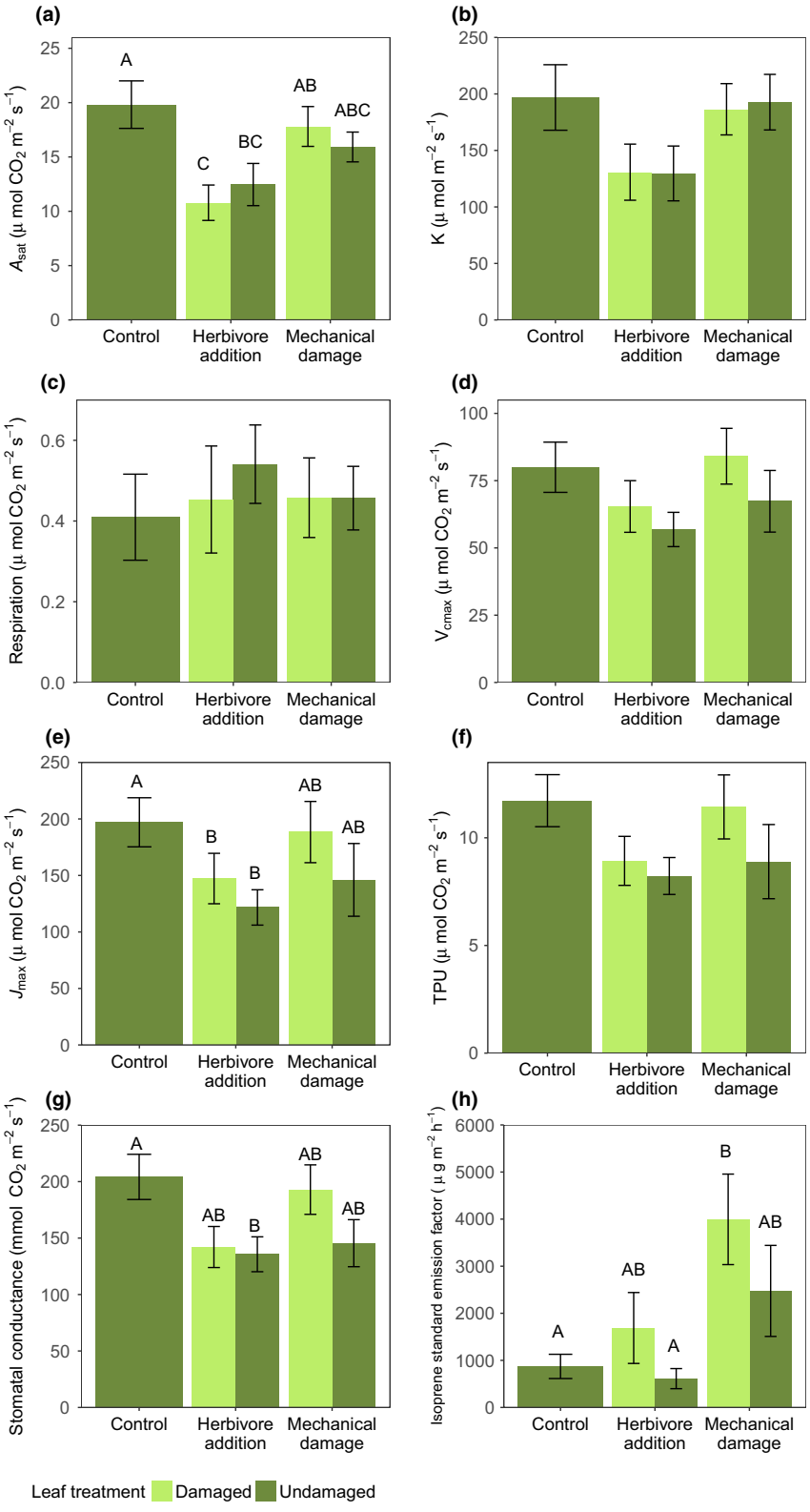

Fig. 2 The average parameter values per leaf treatment on leaves of *Quercus robur* subject to herbivory by *Operophtera brumata*, mechanical damage or left as intact (control). (a) The average maximum model-fitted light-saturated photosynthetic rate (A_{sat}), (b) the average light intensity at which the model-fitted photosynthetic rate is half of its maximum (K), (c) the average dark respiration rate (R_d), (d) the temperature-corrected average maximum carboxylation rate (V_{cmax}), (e) the temperature-corrected average maximum electron transport rate (J_{max}), (f) the temperature-corrected average triose phosphate use efficiency (TPU), (g) the average stomatal conductance (g_s) and (h) the average standard isoprene emission rate (I_S). $n = 10$ per leaf treatment for the figures in (a–c), except $n = 9$ for the mechanically damaged leaf and $n = 9$ for herbivore undamaged leaf for (b). For figures in (d–f), $n = 15$ for control, $n = 13$ for the herbivory treatments and $n = 12$ for the mechanical treatments. For (g) $n = 19$ for control, $n = 18$ for damaged leaf in herbivore treatment and intact leaf in mechanical treatment, and $n = 17$ for intact leaf in the herbivore treatment and damaged leaf in the mechanical treatment. For (h) $n = 7$ for control and damaged leaf in the mechanical treatment, $n = 6$ for undamaged leaf in the mechanical treatment and intact leaf in the herbivory treatment, and $n = 4$ for the damaged leaf in the herbivory treatment. Error bars are ± 1 SEM. Means not sharing a letter are statistically significantly different from one another, e.g. AB and C in (a) (Tukey's test, $P < 0.05$). Note that the y-axis for respiration (c) is expressed as positive values (instead of the negative assimilation rates) to make the graph more intuitive. The data represent measures from both field sites, and in (d–g) during both measuring years. Note that the effect of site and year has been taken into account in the statistical analyses.

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Table 1 Total effect of the herbivory by *Operophtera brumata* on *Quercus robur* from the leaf to the canopy scale

	Intact leaf, intact shoot ($t = 1$)	Intact leaf, damaged shoot ($t = 2$)	Damaged leaf, damaged shoot ($t = 3$)	Canopy scale total effect
Direct effect				
Leaf area loss (%) (D_t)	0	0	−8.5 ± 0.4	
% of leaves in canopy (l_t)	1.7	27.3 ± 1.9	71.0 ± 1.9	
Canopy-scale effect % (D_c)				−6.0 ± 3.8
Light saturated photosynthesis (A_{sat})				
Rate (μmol CO_2 m^{-2} s^{-1} of leaf area)	19.8 ± 2.2	12.5 ± 1.9	10.8 ± 1.6	
Rate (% of intact)	100	63.1 ± 11.9	54.5 ± 10.1	
Indirect effect per unit leaf area %	0	−36.9 ± 11.9	−45.5 ± 10.1	
Leaf-scale effect % (direct + indirect)$^{Eqn\ 1.}$	0	−36.9 ± 11.9	−50.1 ± 9.5	
Canopy-scale effect % (direct + indirect)$^{Eqn\ 2.}$				−45.6 ± 7.60
Isoprene				
Rate (μg m^{-2} h^{-1} of leaf)	871.7 ± 257.6	612.1 ± 213.5	1766.0 ± 967.0	
Rate (% of intact)	100	70.2 ± 32.1	202.6 ± 126.0	
Indirect effect per unit leaf area %	0	−29.8 ± 32.1	102.6 ± 126.0	
Leaf-scale effect % (direct + indirect)$^{Eqn\ 1.}$	0	−29.8 ± 32.1	85.4 ± 115.6	
Canopy scale effect % (direct + indirect)$^{Eqn\ 2.}$				52.5 ± 82.6
Light diffused photosynthesis				
Canopy net rate per leaf type (μmol CO_2 m^{-2} s^{-1} of ground area, NPC_t)$^{Eqn\ 3}$	29.96 ± 3.19	17.87 ± 2.59	16.92 ± 2.28	
Canopy net rate combined, weighted with the leaf type proportions (μmol CO_2 m^{-2} s^{-1} of ground area)				17.4 ± 1.83
Canopy net rate (% of intact)				58.1 ± 8.70
Canopy-scale effect % (direct + indirect)$^{Eqn\ 4}$				−47.9 ± 9.50

The average percentage of leaf area loss per leaf (D_t, direct effect), the average proportion of different leaf types ($t = 1, 2, 3$) in the canopy, the effect of insect herbivory on the light-saturated photosynthetic rate (A_{sat}) and on the isoprene emission rate per unit leaf area (indirect effect) of the different leaf types, the estimates of the combined (direct + indirect) effects of these at leaf and canopy scales, and the canopy-scale estimates when change in the light intensity through the canopy is taken into account. The effects are expressed relative to the control treatment values (intact leaves in intact shoots). Errors are ± 1 SEM derived through error propagation. See Supporting Information Table S3 for values for photosynthetic rate in 1000 μmol m^{-2} s^{-1} of photosynthetically active radiation (A_{1000}).

effects of herbivory on plant physiology, which can be visible several weeks (Gibberd *et al.*, 1988; Meyer, 1998) or even seasons (Kaitaniemi *et al.*, 1998) after the initial damage.

One possibility is that physical injury is inhibiting photosynthesis. Severed vein network can disrupt the transport of water and nutrients with long-lasting effects (Sack & Holbrook, 2006), simultaneously reducing stomatal conductance. Ruptures in the leaf can cause diffusion of CO_2 before it is used in the carbon (C)-fixing reactions, lowering the efficiency of C assimilation (Oleksyn *et al.*, 1998; Nabity *et al.*, 2006, 2009, 2013). Furthermore, repairing the damaged tissue uses valuable resources. Trade-offs in resource use might also occur between growth (and hence photosynthesis) and defence (Herms & Mattson, 1992). Defensive reactions against herbivores require synthesis of complex chemical compounds, which act as repellents or additional signalling molecules, using the same resources or molecular pathways than photosynthesis (Herms & Mattson, 1992; Taiz & Zeiger, 2010; Zhou *et al.*, 2015). Build-up of defensive compounds in the plant tissue also might cause the problem of autotoxicity, lowering photosynthetic efficiency (Baldwin & Callahan, 1993; Nabity *et al.*, 2009). Damage early in the season also could 'prime' the plant (Conrath *et al.*, 2002), making it more resistant to future herbivory by activating long-lasting defences. The cost of maintaining a primed state could alter primary metabolism over the long term (van Hulten *et al.*, 2006; Frost *et al.*, 2008).

Why does the photosynthetic rate differ between leaves damaged mechanically or by herbivores?

In the present study, the mechanically damaged leaves experienced a significantly smaller reduction in their photosynthetic rate than leaves damaged by caterpillars. In previous studies, mechanical damage alone has failed to produce a response in the plant, whereas application of herbivore oral secretions, even without any physical damage, have done so (Alborn, 1997; Korth & Dixon, 1997). The herbivore-induced defensive responses depend on the species identity, specifically on the chemical make-up of the insect saliva (Alborn, 1997; Erb *et al.*, 2012). These herbivory-specific effects are usually mediated through hormonal pathways including jasmonic and salicylic acids, the activation of which also switches off photosynthesizing reactions (Wasternack & Hause, 2013). These results suggest that the herbivory-inflicted photosynthetic reduction in our study is a response to the presence of herbivores specifically, instead of leaf damage alone, and possibly actively triggered by the defence machinery of the plant (Kerchev *et al.*, 2012; Zhou *et al.*, 2015).

How does leaf damage affect intact neighbouring leaves?

In the present study, intact and damaged leaves on the same shoots showed an almost identical degree of reduction in

photosynthesis. Damage-triggered defence reactions can travel to intact plant parts through shared vasculature (Jones *et al.*, 1993), as electric signals (Sukhov, 2016), or to neighbour plants through volatile organic compounds (Arimura *et al.*, 2000). This systemic signalling can subsequently affect photosynthesis of intact plant parts (Agrawal, 2000; Barron-Gafford *et al.*, 2012; Meza-Canales *et al.*, 2017). Especially jasmonic acid can travel to systemic tissues (Baldwin & Zhang, 1997; Stratmann, 2003), and accumulate in them (Leitner *et al.*, 2005). Because in our study the systemic changes were detected within individual shoots, the signal has probably travelled through within-shoot vascular connections, which also might have restricted it from reaching the intact control shoots, or dampened the effect (Orians, 2005). The reduction in photosynthesis in neighbouring leaves might prepare the leaf for the forthcoming herbivory, either by increasing the level of defences at the expense of assimilation, or by actively shutting down the production of further carbohydrates, to provide less nutrition for herbivores (Zhou *et al.*, 2015). Herbivore-specific signalling might also explain why the mechanical treatment responded less than the herbivore addition. Our study thus shows that naturally occurring herbivory can have a considerable effect also on systemic intact leaves. These kinds of shoot-level effects have not been previously taken into account in ecosystem-scale studies.

Why did the isoprene emission rate increase after leaf damage?

We observed a significant positive relationship between photosynthesis and isoprene emission, concurrent with previous studies (Rasulov *et al.*, 2009; Copolovici *et al.*, 2017). Nevertheless, the treatment-specific effects on isoprene were opposite to the effects on photosynthesis. The isoprene emission rates per unit leaf area were significantly higher in the mechanically damaged leaves than in nondamaged leaves on the intact control shoots, suggesting that the observed change might not be a response to herbivory specifically. Because the effect was not visible in the surrounding intact leaves, the damage-triggered change in isoprene emission seems to be a leaf-level response. Contrary to our results, previous studies have found *a reduction* in isoprene emission immediately after leaf damage (Loreto & Sharkey, 1993; Portillo-Estrada *et al.*, 2015; Copolovici *et al.*, 2017; but see Ferrieri *et al.*, 2005). VOC emission profile emitted immediately after damage can substantially differ from longer-term emissions (Maja *et al.*, 2014). Nevertheless, most herbivore-induced VOCs are studied immediately after the damage occurs.

Oak could be actively increasing its isoprene emission over a longer period after the damage. Physical injury to the leaf venation network could lead to increased water loss lasting for several days (Aldea *et al.*, 2005). Drought, and a release from it, have been shown to increase isoprene emissions (Sharkey & Loreto, 1993; Tattini *et al.*, 2015). If mechanical damage caused water stress at the time of the injury, this might have led to increased isoprene emission later, once the damage had been repaired. Long-term monitoring of damage-induced isoprene emission is needed to fully understand its response to herbivory.

Canopy-scale effect of insect herbivory

At our study site, on the one hand the *direct effect* of insect herbivory was small: insect herbivores removed 6.0% (± 3.8%) of the oak leaf area, consistent with global estimates of average herbivory rates (Cyr & Pace, 1993). On the other, the *indirect effect* of herbivory on the remaining leaf tissue of the damaged leaf, and on the neighbouring intact leaves, was several magnitudes larger, reducing the light-saturated photosynthesis by 46% (± 10%) and 37% (± 12%) on average, respectively. This supports the previous results on the importance of indirect effects over direct ones (Zangerl *et al.*, 2002; Barron-Gafford *et al.*, 2012). Nevertheless, in many ecosystem-scale studies the effects of herbivory are quantified only as the amount of leaf area loss (Metcalfe *et al.*, 2014).

By combining indirect effects with the leaf area loss (8.5% ± 0.4% per leaf), we estimate that every damaged leaf has its photosynthetic rate reduced by 50% (± 10%). Surveying the natural intensity of herbivory in the area, only 1.7% of shoots per tree were completely intact. Therefore, most of the oak canopy (98.3%) is photosynthesizing below its potential. Effectively no tree in natural settings is devoid of this herbivory-influenced suppression of photosynthesis. On a scale of the canopy, then, only 52% (± 10%) of the photosynthesis is realized. Previous studies have not considered the combined direct and indirect effects on the ecosystem-level C cycle. We show that herbivores can reduce canopy-scale C sequestration considerably, and the shoot-level effect observed in the intact neighbour leaves is a major contributor to this reduction.

Similarly, herbivory had a large effect on isoprene emission, causing an 85% (± 116%) increase in the leaf-scale isoprene emission rate and a 53% (± 83%) increase at the canopy scale. The large error margin makes it difficult to draw firm conclusions on the role of herbivory on canopy-level isoprene emissions. However, if our estimates are correct, this increase would be enough to counteract the predicted reduction in isoprene emissions due to climate change, increasing atmospheric CO_2 concentrations and land-use changes combined (Squire *et al.*, 2014). Despite their potential importance, biotic interactions are usually lacking from the global isoprene emission models (Arneth *et al.*, 2008; Müller *et al.*, 2008; Squire *et al.*, 2014). Previous studies have recorded higher forest-scale isoprene emissions than expected by models (Geron *et al.*, 1997; Gu *et al.*, 2017), and changes in species composition have been shown to affect forest-scale isoprene emissions (Wang *et al.*, 2017). Our study suggests that enhanced emissions resulting from leaf damage might be leading to underestimates of the actual forest-scale isoprene emissions, which could have significant knock-on effects on calculations of ozone and particle formation.

Because emission of isoprene is temperature-sensitive, measurements of temperature change through the different canopy layers would be needed for a more realistic estimate on canopy-level isoprene emissions. Also, further studies on differences between sun and shade leaves and herbivory rates across the canopy, and direct canopy measurements are needed to improve the estimates on canopy photosynthesis and isoprene emissions under herbivory.

With the predicted climate change, species distributions, abundances and hence the frequencies of specific species interactions are projected to shift, and in many cases, have already shifted (Jepsen *et al.*, 2008; Kurz *et al.*, 2008). Nevertheless, insect herbivory is rarely addressed in biosphere and climate models (Kurz *et al.*, 2008). Our results clearly demonstrate that for predicting the responses of forest ecosystems to climate change, including the effects of herbivory on the C cycle and atmospheric chemistry is crucial. Ignoring the role of insect herbivory might thus overestimate the role of forests as C sinks (Kurz *et al.*, 2008; Schäfer *et al.*, 2010), or underestimate their role as isoprene emitters. We have demonstrated the importance of indirect herbivory effects for a single plant–herbivore system; there is a clear need to replicate such studies in other systems.

Conclusions

Moth caterpillars reduce the per unit leaf area photosynthetic rate of their host plant, both in the remaining leaf tissue of the damaged leaf, and in the intact neighbour leaves. The reduction by natural herbivory is greater than that by mechanical damage alone. This indicates that the host plant can differentiate between these two types of damage, pass on the signal to undamaged parts and respond accordingly. Isoprene emission rate is increased by mechanical leaf damage, and does not seem to be a herbivory-specific reaction. These responses expressed on a scale of individual leaves and shoots have large-scale consequences on the C dynamics on the scale of the forest. At the scale of a canopy, the indirect effects of herbivory emerge as several times more important than the direct effect of leaf area removed. Including these effects in estimates of the interactions between biosphere and the atmosphere is crucial for better prediction of the effects of changing climate on forest ecosystems.

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Author contributions

K.V., S.G. and T.R. designed the research; K.V. carried out the data collection, analysed the data and wrote the first version of the paper; S.G., T.R. and Y.M. supervised the writing and analyzing; C.B., I.O. and S.R. contributed to the data analyses; C.B. and N.H. designed the isoprene measuring system; and all authors contributed substantially to revisions. S.G. and T.R. are joint senior authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Example of a mesh bag.

Fig. S2 Experimental leaves in herbivory addition and mechanical damage –treatments.

Fig. S3 The average *A*/*C*i response curves per leaf treatment.

Fig. S4 Correlation between the isoprene emission rate and photosynthetic parameters.

Table S1 Leaf area loss at the study area and in the experiment

Table S2 Coefficient estimates for mixed effects models

Table S3 Effects of herbivory on A_{1000} on leaf and canopy scales

Methods S1 Details on the experimental set up and on extracting the gas exchange parameters.

Methods S2 iDirac overview and operation.

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