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Oak gall wasp infections of *Quercus robur* leaves lead to profound modifications in foliage photosynthetic and volatile emission characteristics

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Abstract

Oak trees (*Quercus*) are hosts of diverse gall-inducing parasites, but the effects of gall formation on the physiology and biochemistry on host oak leaves is poorly understood. The influence of infection by four species from two widespread gall wasp genera, *Neuroterus* (*N. anthracinus* and *N. albipes*) and *Cynips* (*C. divisa* and *C. quercusfolii*), on foliage morphology, chemistry, photosynthetic characteristics, constitutive isoprene and induced volatile emissions in *Q. robur* was investigated. Leaf dry mass per unit area (M_A), net assimilation rate per area (A_A), stomatal conductance (g_s), and constitutive isoprene emissions decreased with the severity of infection by all gall wasp species. The reduction in A_A was mainly determined by reduced M_A and to a lower extent by lower content of leaf N and P in gall-infected leaves. The emissions of lipoxygenase pathway (LOX) volatiles increased strongly with increasing infection severity for all four species with the strongest emissions in major vein associated species, *N. anthracinus*. Mono- and sesquiterpene emissions were strongly elicited in *N. albipes* and *Cynips* species, except in *N. anthracinus*. These results provide valuable information for diagnosing oak infections using ambient air volatile fingerprints and for predicting the impacts of infections on photosynthetic productivity and whole tree performance.

Keywords

Cynips; gall wasps; indirect defenses; induced volatile emission; isoprene; monoterpenes; *Neuroterus*; photosynthesis; quantitative responses

Introduction

Gall wasps are a tribe of obligate parasites on plant leaves (Stone *et al.* 2002). The formation of specialized tumor-like sinks on the leaf surface is an extremely complex mutual interaction between gall wasps and the host plant (Taper & Case 1987). By redirection of the

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growth and physiology of the infected plant organs for their own benefit, the secretion from the ovipositioning female creates galls that provide carbon, nutrients and protection against predators or adverse weather conditions for the larva (Nylander 2004; Mani 1992; Nyman & Julkunen-Tiitto 2000; Raman *et al.* 2007; Stone *et al.* 2002; Stone & Schönrogge 2003).

The infection by gall-inducing parasites results in major alterations in morphology, physiology, and biochemistry of their host plants (Albert *et al.* 2011; Tooker *et al.* 2008). The competition for leaf photosynthetic products between gall consumption for larval benefits and leaf development (Abrahamson & Weis 1987; Bronner 1992; McCrea *et al.* 1985) leads to the changing patterns of plant biomass accumulation and photosynthetic rates (Fay *et al.* 1996; Hess 1986; Larson 1998; Prior & Hellmann 2010; Tooker & De-Moraes 2008; Washburn 1984). The initiation, growth and maintenance of galls on plant organs (leaves, stems, fruits, and buds) can alter multiple host traits, including plant architecture (Larson & Whitham 1997), shoot growth (Hartnett & Abrahamson 1979; Vuorisalo *et al.* 1990), and nutrient allocation (Abrahamson & McCrea 1986; McCrea *et al.* 1985), and ultimately impact whole-plant growth and survival (Hakkarainen *et al.* 2005). Regarding physiology, gall formation has been found to modify foliar gas-exchange characteristics, including photosynthesis, stomatal conductance for water vapor, and water use efficiency (Dorchin *et al.* 2006; Fay *et al.* 1993; Florentine *et al.* 2005; Larson 1998), although the magnitude of effects vary among gall-host systems. For example, large declines in photosynthetic capacity (approx. 60%) and stomatal conductance (approx. 50%) in the canopy leaves of mature sugar maple (*Acer saccharum*) trees galled by the maple spindle gall mite *Vasates aceriscrumena* have been reported (Patankar *et al.* 2011), while the photosynthesis in *Machilus thunbergii* leaves was not significantly affected by the number of cecidomyiid insect galls which themselves have a certain photosynthetic activity (Huang *et al.* 2014). Collectively, the studies suggest that the impacts of gall formation on gas-exchange processes are variable and likely determined by the type of gall inducer attacking the host plant (Welter 1989).

Gall-forming insects can also alter host-plant chemistry and the distribution of plant secondary metabolites for their own benefit, including the modification of the volatile composition of host-plant cues used to attract mates or deter natural enemies (Augustyn *et al.* 2010; Hartley 1998; Nyman & Julkunen-Tiitto 2000; Tooker *et al.* 2005; Tooker & Hanks 2004; Allison & Schultz 2005; Augustyn *et al.* 2015). It has been reported that the infection of the tephritid fly *Eurosta solidaginis* and the gelechiid moth *Gnorimoschema gallaesolidaginis* galls can influence indirect plant defenses by manipulation of the volatile emission from the host, goldenrod (*Solidago altissima*) (Tooker *et al.* 2008). However, volatile organic compounds, especially terpenoids, were induced from *Dittrichia viscosa* flowers infected by the gall-forming dipteran *Myopites stylatus*, which in turn facilitated the parasitism by hymenopteran species (Santos *et al.* 2016). Although there are several case studies of gall-induced modifications in host plant secondary chemistry, there is a paucity of studies about the effects of different gall species on the volatile profiles of the same host plant species. There is also overall lack of information about quantitative relationships between the infection severity and leaf physiological response, prompting our study to explore the responses of volatile emission to the infection of gall-inducers.

Pedunculate oak (*Quercus robur* L.) is one of the most susceptible tree species to infection by diverse specialized gall wasp species (Stone *et al.* 2002). Among more than 1000 species of oak gall wasps (Stone *et al.* 2002; Liljeblad *et al.* 2008), *Neuroterus* and *Cynips* taxa are listed as the most diverse gall wasp genera globally, and these genera are widely distributed in Europe (Abrahamson & Weis 1987; Ronquist & Liljeblad 2001), and under favorable conditions, mass gall infections are common (Agrios 2005). Studies on the structure of the galls have demonstrated that both the *Neuroterus* and *Cynips* galls contain several layers of epi- and hypodermal and parenchymatous tissues including a protective outer layer and an inner layer of nutritive tissue (Stone & Schönrogge 2003), and they sequester nutrients from the surrounding leaf tissue to the nutritive tissue of the gall (McCrea & Abrahamson 1985; Bronner 1992). However, whether the consumption of photosynthetic products and nutrients of the host leaves for oak gall formation leads to a reduction of physiological activity of the infected leaves and major losses of biomass yield is unknown.

Quercus robur is a moderately strong isoprene-emitting oak species (Schnitzler *et al.* 1997; Copolovici *et al.* 2014c). Although the release of volatiles including isoprene, mono- and sesquiterpenes, volatile products of the lipoxygenase pathway (LOX, also called green leaf volatiles) and benzenoids can be elicited by pathogenic fungi and herbivores in *Q. robur* leaves (Copolovici *et al.* 2014c; Ghirardo *et al.* 2012; Copolovici *et al.* 2017), it is unknown whether the oak gall wasps are inducers of volatile emissions as well, and how different gall wasp species alter the volatile emissions. If they do, it is further necessary to know whether the severity of the gall infection (generally evaluated by the size or number of galls) is quantitatively related to the emission rates of constitutive and induced volatiles, but also to the photosynthetic traits. However, to our knowledge the quantitative relationships between the severity of gall infection and the emission rates of induced volatiles have not been studied.

This study tested the hypotheses that oak gall infections lead to a reduction in foliage photosynthesis and constitutive volatile emissions and elicit stress volatile emissions in an infection severity-dependent manner in *Q. robur*, and that infections by different wasp species lead to different quantitative relationships and different induced emission blends. We studied the effects of infection by four species of gall wasp from the *Neuroterus* (*N. anthracinus* and *N. albipes*) and *Cynips* (*C. divisa* and *C. quercusfolii*) genera on foliage photosynthetic characteristics and on constitutive and induced volatile emissions. The results indicate that both *Neuroterus* and *Cynips* gall infections do induce alterations in photosynthesis and constitutive isoprene and induced volatile (LOX, monoterpenes) release that scale quantitatively with the infection severity in oak. Different quantitative relationships between the severity of gall infection and volatile release among leaves infected by different gall species were observed, highlighting the complexity of host-gall interactions even in the same host species.

Materials and Methods

Sampling site and plant material

The study was conducted at Tartu Tammik, Ihaste, Tartu, Estonia (58.36°N, 26.77°E, elevation 40 m above sea level). The site supports a sparse *Q. robur* plantation (ca. 100 trees

ha⁻¹). The plantation was established in 2007 with 7-8 years old and 3-4 m tall trees, and by the time of sampling in 2015-2016, the trees were 15-16 years old and 6-8 m tall. Both study years were characterized by a cool and humid summer with a monthly mean ($\pm$ SE) precipitation of 48.5 ± 5.4 mm (2015) and 88.6 ± 25.2 mm (2016), air temperature of 15.5 ± 1.0 °C (2015) and 16.1 ± 1.2 °C (2016), and relative air humidity of $73.2 \pm 3.7\%$ (2015) and $76.1 \pm 2.9\%$ (2016) from June to September (data of Laboratory of Environmental Physics, Institute of Physics, University of Tartu, Tartu, Estonia, <http://meteo.physic.ut.ee>).

Galls of different species were observed from mid-July to September, but the severity of infection of *Q. robur* leaves by different gall wasp species varied in different years. In particular, the year 2015 was characterized by a mass infection of *Neuroterus anthracinus* with most leaves infected, while in 2016 *N. anthracinus* infections were relatively infrequent. In 2016, the key infecting species was *N. albipes* that was seldom observed in 2015. The infections by *Cynips* species (*C. divisa* and *C. quercusfolii*) were diffusely spread in both years. Thus, the collection and measurement of *Q. robur* leaves infected by *N. anthracinus* was conducted in the beginning of September 2015, while the leaves infected by *N. albipes* and the *Cynips* species (*C. divisa* and *C. quercusfolii*) were studied in the beginning of September 2016. As the cross-sections of the galls demonstrated, the hatched larvae were in a similar developmental state in most cases, except for *N. albipes* where leaves could carry galls with different developmental state (Fig. 1b, c). As the infections were spread to all trees at the site, for controls we used non-infected leaves in the infected trees in both years. Although there might be systemic effects also affecting these non-infected leaves, we note that the photosynthetic rates, and constitutive and induced volatile emission rates in these trees were similar to leaves in completely non-infested trees (Niinemets *et al.* 2010; Copolovici *et al.* 2014b; Copolovici *et al.* 2014c; Copolovici *et al.* 2017).

For each species, foliage was collected from at least five different *Q. robur* trees, and leaves with varying gall number and gall mass were collected for the measurements. About 20 cm long twigs with multiple leaves were sampled from the outer exposed surface of tree crown for physiological measurements in the laboratory. The twigs were excised under water, and with the cut ends kept submerged, the twigs were immediately transported to the laboratory where a representative leaf was selected for measurements. Studies demonstrate that use of cut twigs is a valid method for photosynthesis (Lange *et al.* 1986), and our previous measurements also demonstrate that comparable photosynthesis and volatile emission measurements can be obtained with attached (Niinemets *et al.* 2010) and detached (Copolovici *et al.* 2014c; Copolovici *et al.* 2017) shoots in *Q. robur*. For *N. anthracinus* and *N. albipes* infections, 23 leaves with different degrees of infection were measured in both cases, and for infections by *Cynips* species 10 leaves infected by *C. divisa* and 16 leaves infected by *C. quercusfolii* were measured (Fig. 1). In each case, three non-infected leaves were measured (controls).

Identification of *Neuroterus* and *Cynips* gall species

The galls were cut in half with a razor blade and the gall diameter was measured. Freehand sections were made from the galls, stained with toluidine blue and viewed with a Nikon

Eclipse E600 microscope and then photographed with a Nikon 5 MP digital microscope camera DS-Fi1 (Nikon Corporation, Kyoto, Japan). From these images, the gall-wall thickness was measured. For anatomical analyses, gall material was infiltrated in a fixation buffer (3% glutaric aldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH = 6.9) under vacuum in a syringe. The samples were post-fixed for 1 h in an osmium tetroxide solution (2%). They were further dehydrated in a series of increasingly stronger ethanol solutions and embedded in LR white resin (Electron Microscopy Sciences, Hatfield, PA, USA) according to standard procedures (Tosens *et al.* 2012). Subsequently, the samples were polymerized in an oven at 60°C for 26 h. Semi-thin cross-sections of 1 µm were cut and stained with toluidine blue for light microscopy. The stained semi-thin sections were viewed in brightfield with a Nikon Eclipse E600 microscope with phase contrast at magnifications of 100×, 200× and 400×, and photographed with a Nikon 5 MP digital microscope camera DS-Fi1 (Fig.1f-i). The quantitative and qualitative information obtained from freehand and microtome sections was used to identify the gall species according to an oak gall identification key (Williams 2006).

Foliage gas-exchange measurements

A custom-made gas-exchange system (Copolovici & Niinemets 2010; Copolovici *et al.* 2014; Jiang *et al.* 2016 for details) was used to measure foliage net assimilation and transpiration rates and emissions of volatiles from *Q. robur* leaves with varying degree of gall infection. The system used has a 1.2 L temperature-controlled double-walled glass chamber with glass and stainless steel bottom. Ambient air (mean $\pm$ SE CO₂ concentration of 402 $\pm$ 28 µmol mol⁻¹) was drawn from outside with a pump and passed through a custom-made ozone scrubber and a humidifier (Copolovici & Niinemets 2010; Copolovici *et al.* 2014). The flow rate was maintained at 1.76 L min⁻¹, and turbulent conditions in the chamber were obtained by a fan installed inside the chamber. For the first-order decay kinetics, the half-time of the chamber air exchange was estimated to be approximately 30 s (Niinemets *et al.* 2011). Leaf temperature was measured with a thermocouple attached to the lower leaf surface. The standard measurement conditions used in these measurements were: light intensity at the leaf surface of 700 µmol m⁻² s⁻¹, leaf temperature of 25 °C and relative air humidity of 60%. Concentrations of CO₂ and H₂O in the incoming and outgoing air were gauged with an infra-red dual-channel gas analyzer operated in the differential mode (CIRAS III, PP-systems, Amesbury, MA, USA).

After the enclosure of the leaf, standard measurement conditions were established and the leaf was conditioned until leaf gas-exchange rates reached a steady state (typically in 20-30 min after the enclosure). Thereafter, the steady-state rates of net assimilation and transpiration were recorded and air samples for estimation of volatile organic compound emissions were taken (see the next section).

Analysis of volatile emissions by gas chromatography-mass spectrometry

Air samples for estimation of volatile emission were drawn from the gas-exchange chamber by an air sampling pump (210-1003MTX, SKC, Inc., Houston, TX, USA; Niinemets *et al.* 2011) operated with a flow rate of 0.2 L min⁻¹ for 20 min. Stainless steel cartridges filled with three carbon-based adsorbents with different specific surface area were used for

sampling to trap all the volatiles in the C3–C15 range (Kännaste *et al.* 2014). In addition to intact control and infected leaves, also emissions from infected leaves with galls removed, and from separated galls were measured. In the latter measurements, the volatiles were sampled in 20 min after gall removal to avoid interfering emissions of volatiles due to leaf wounding right after the damage. This fast wounding response stops within 8–10 min. after leaf wounding (Portillo-Estrada *et al.* 2015). Due to the lack of stomata on the gall surface and thick waxy cuticle (Fig. 1), the loss of water and changes in physiological activity of galls during this conditioning period was considered minor. We determined the fresh mass of the galls just after removal and after volatile measurements, and observed that the change of the mass was negligible (less than 0.1%). In all cases, blank chamber samples were taken before and after sampling of plant material.

The cartridges were analyzed by a Shimadzu 2010 Plus gas chromatograph equipped with a mass spectrometric detector (GC–MS) (Shimadzu Corporation, Kyoto, Japan), and with a Shimadzu TD20 automated cartridge desorber. A Zebron ZB-624 fused silica capillary column (0.32 mm i.d.×60 m, 1.8 µm film thickness, Phenomenex, USA) was used for GC–MS analysis according to the protocol of Kännaste *et al.* (2014). For compound identification, compound retention indices, and the mass spectra of volatiles of the authentic standards and the spectra available in the National Institute of Standards and Technology library (NIST 05) were used and the GC–MS system was calibrated with the authentic standards as described before (Copolovici *et al.* 2009; Kännaste *et al.* 2014; Jiang *et al.* 2016; Jiang *et al.* 2017).

Analysis of N, C, P contents of the galls and infected leaves

A subset of non-infected leaves (control, $n = 3$) and severely gall-infected leaves of *N. anthracinus*, *N. albipes* and *C. quercusfolii* ($n = 3$ for each species) were collected for chemical analyses. The galls were mechanically separated from the leaves and chemical analyses were conducted for the control leaves, infected leaves without galls and for the separated galls of *C. quercusfolii* (there was not enough gall material for the galls formed by the two other gall-forming wasp species). The contents of carbon (C) and nitrogen (N) were estimated by a Vario MAX CNS analyzer (Elementar Analysensysteme GmbH, Hanau, Germany), and phosphorus (P) content was estimated by microwave plasma atomic emission spectroscopy (Agilent MP-AES 4100) after sample digestion in sulphuric acid.

Data analyses

The traits characterizing gall infection, gall size, number of galls and gall area per leaf area and gall dry mass per total leaf dry mass (M_g/M_l) were compared among different galling species by ANOVA. ANOVA was also used to compare leaf structural and photosynthetic characteristics and volatile emission rates among control and severely infected leaves. The ratio M_g/M_l was used as a quantitative measure of the severity of infection, and the statistical relationships of leaf structural, photosynthetic characteristics and emission rates of volatiles with M_g/M_l were explored by linear and non-linear regression analyses. The statistical functions yielding the greatest degree of explained variance (r^2) were reported. In these analyses, all leaves from different trees were pooled. In the case of *Cynips* species, the differences in M_g/M_l vs. leaf trait relationships were generally small among different species

(in general, not significantly different according to co-variation analyses), and both species were pooled in the regression analyses. These statistical analyses were conducted with SAS (Version 8.02, SAS Institute, Cary, NC). Differences in bivariate leaf trait relationships among infecting gall species (slope and elevation differences) were separated by standardized major axis tests using Smatr ver. 2.0 (Falster *et al.* 2003). Principal component analysis (PCA) of volatile emissions from the leaves infected by different gall-forming wasps was performed by SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) as in Kännaste *et al.* (2014). All statistical effects were considered significant at $P < 0.05$.

Results

General characteristics of and overall frequency of infection by *Neuroterus* and *Cynips* galls

The morphological characteristics (including the size, color and texture) widely varied among different oak gall species (Fig. 1a-e). In addition, in *N. albipes*, the color of galls ranged from creamy white to reddish, and typically, creamy galls were immature and smaller (Fig. 1b, f), while mature galls showed red color and were bigger (Fig. 1c, g). In *Cynips*, *C. quercusfolii* galls were light green (Fig. 1d, h), while *C. divisa* showed yellow color (Fig. 1e, i). Compared to the galls of *Cynips* species, the galls of *N. albipes* were denser and had smaller cells (Fig. 1f-i). The galls of *N. anthracinus* and *Cynips* species were always found on the lower leaf surface, while *N. albipes* galls were occasionally (in the case of ca. 5-10% of infected leaves) also found on the upper leaf surface (Fig. 1a). The galls of *N. anthracinus* were typically associated with the main veins, and less frequently with second order major veins (Fig. 1a). The galls of *Cynips* were typically attached to the leaf lamina, although often in the proximity of major veins, and the galls of *N. albipes* were typically associated with intercostal leaf areas (Fig. 1a).

Quantitative analyses also confirmed the wide structural variation of the galls induced by different wasp species (Table 1). In particular, the overall size of the galls, area and dry mass increased in the sequence *N. anthracinus* < *N. albipes* < *C. divisa* < *C. quercusfolii* (Table 1). As the ultrastructural data suggested (Fig. 1f-i), dry to fresh mass ratio was indeed bigger for *Neuroterus* than for *Cynips* galls (Table 1) in agreement with the greater tissue density of *Neuroterus* galls.

Compared with the galls of *Neuroterus* spp., in leaves infected by *Cynips* spp. the number of galls per leaf was smaller (Table 1). However, due to their greater size, the overall percentage of leaf area covered by galls and gall to leaf dry mass ratio (M_g/M_l) were greater for *Cynips*-infected than for *Neuroterus*-infected leaves (Table 1).

Estimation of the severity of leaf infection by *Neuroterus* and *Cynips* gall wasps

Infection by *N. anthracinus* resulted in brownish or yellowish necrotic and senescent leaf areas distal to the infection sites at the major veins (Fig. 1a). Although *Cynips*- and *N. albipes*-infected leaves did occasionally have some dried and chlorotic leaf spots at leaf margins (up to 5-10% of total leaf area) contributing to their overall unhealthy appearance (Fig. 1a), these areas were typically not directly connected through major veins to the gall

attachment. In addition, not all necrotic leaf area in *N. anthracinus*-infected leaves was directly associated with galls (Fig. 1a). As direct damage that could be causally linked to gall infection was difficult to estimate, and the boundary between dark green and pale areas was not well demarcated, objective characterization of the damage percentage of the leaf area due to galls was impossible. Therefore, we decided to use the overall level of infection as the proxy of infection severity. The total gall number per leaf showed positive linear or non-linear correlations with dry gall mass in all species (Fig S1a-c). However, the size of galls of a given species for leaves harboring multiple galls and among different leaves varied widely (Table 1 for the coefficients of variation of the size estimates; Fig. 1a-c), and the gall number was weakly correlated with M_g/M_l (Fig. S1d-f). Given the differences in gall size within infecting wasp species, we considered M_g/M_l a more appropriate species-specific proxy of the severity of infection (Table 1 for species differences in average M_g/M_l values) than the number of galls per leaf or per unit leaf area.

Modifications in foliage structure and chemistry upon gall wasp infection

Leaf dry mass per unit area (M_A) was greater in control than in severely infected leaves in all cases, but leaf dry to fresh mass ratio was similar among control and severely infected leaves (Table 2). Across leaves with different degrees of infection, M_A decreased linearly with increasing M_g/M_l in all species (Fig. 2). The reduction was greater for infections by *Neuroterus* species, ca. 2.0–2.6-fold (Fig. 2a, b) compared with *Cynips* spp. infection where M_A was reduced by 1.8-fold for the entire M_g/M_l range (Fig. 2c, $P < 0.001$ for slope differences among *Cynips* and two *Neuroterus* species, whereas the slopes did not differ among *Neuroterus* species, $P > 0.9$).

Analyses of differences in elemental content of control non-infected leaves and leaves with galls removed indicated that the gall-forming wasp infection did not affect leaf C content per dry mass, but leaf N content per dry mass was lower for leaves infected by *C. quercusfolii*, and leaf P content per dry mass was lower for leaves infected by *N. anthracinus* (Table 2). Due to lack of sufficient material, elemental contents were not analyzed for galls of *Neuroterus* spp., but for *Cynips* galls, average ($\pm$ SE) gall C content ($34.4 \pm 1.3\%$) was 1.2-fold, N content ($0.340 \pm 0.010\%$) 5.4-fold and P content ($0.050 \pm 0.007\%$) 8.3-fold lower than corresponding averages for leaves with galls removed (Table 2 for the leaf data; means for all three chemical elements are significantly different among galls and leaves at $P < 0.01$).

Photosynthetic characteristics and constitutive isoprene emissions of wasp-infected leaves of *Q. robur*

Compared to non-infected control leaves, the light-saturated net assimilation rate per area (A_n) of gall-infected leaves was reduced in all cases (Table 2), and there were negative non-linear hyperbolic (Fig. 3a, b) or close to linear (Fig. 3c) relationships between A_n and M_g/M_l . The reduction was the strongest, 2.7-fold, for *Cynips* spp.-infected leaves, where A_n decreased linearly with increasing infection severity (Fig. 3c). The net assimilation rate per area also decreased strongly in *N. anthracinus*, ca. 2.3-fold, but once the leaves were infected, the reduction was further weakly associated with the infection severity (Fig. 3a). In *N. albipes*, the reduction was 1.8-fold at the highest infection severity, and it also leveled off

with increasing the degree of infection (Fig. 3b). Part of the reduction in net assimilation rate per area was due to a concomitant reduction in M_A , (cf. Fig. 2 and Fig. 3a-c). Nevertheless, net assimilation per dry mass was also lower in gall-infected leaves (Table 2).

Changes in stomatal conductance to water vapor (g_s) due to gall wasp infection were similar to changes in A_n (Table 2; Fig 3d-f), but g_s was generally reduced somewhat less than A_n , and the correlation of g_s with M_g/M_l was non-linear for *Cynips* spp. (Fig. 3i). As the result of smaller changes in g_s , the intercellular CO₂ concentration increased linearly with increasing M_g/M_l in all cases (Fig 3g-i).

Analogously to A_n , the rate of isoprene emission was lower in wasp-infected leaves (Table 3 for the comparison of control and severely-infected leaves), and it decreased curvilinearly with increasing infection severity in all cases (Fig. 4a-c). The reductions in isoprene emission rate were the strongest for *N. anthracinus*, 8.2-fold (Table 3; Fig. 4a) and for *C. quercusfolii*, 7.2-fold (Table 3; Fig. 4c), while in *N. albipes*-infected leaves, isoprene emissions were reduced 4.3-fold (Table 3; Fig. 4b). As the result of the stronger reduction in isoprene emission than A_n , the fraction of carbon going into isoprene synthesis (proportional to isoprene emission rate/ A_n) decreased with increasing the degree of infection (*N. anthracinus*: $r^2 = 0.34$, $P < 0.01$ for a non-linear relationship; *N. albipes*: $r^2 = 0.45$, $P < 0.01$ for a linear relationship; *Cynips* spp.: $r^2 = 0.71$, $P < 0.01$ for a non-linear relationship).

Contrasting responses of stress-induced volatile emissions upon different gall wasp species infection

Monoterpene (including α -pinene, β -ocimene and limonene) emissions of non-infected leaves were very low in both 2015 and in 2016 (Table 3). The emissions of lipoxygenase pathway volatiles (LOX, e.g. (*E*)-3-hexenal and 2-ethyl-1-hexanol) in non-infected leaves were also low in 2016, but the LOX emissions were greater in 2015.

Leaf infection by the four gall species resulted in profound changes in the emission profiles with several species-specific induction responses (Table 3). In all cases the gall infection resulted in greater total LOX emissions (Table 3), and the emissions increased linearly with the infection severity (Fig. 4d-f). Relative to the control leaves, the induction was the greatest for *N. albipes* and *Cynips* spp. infections (Fig. 4e, f), but the greatest absolute elicitation (difference between the infected and control leaves) were observed in *N. anthracinus*-infected leaves (Fig. 4d). The elicitation of terpene and benzenoid emissions upon infection was similar for *N. albipes* and *Cynips* spp. infections (Table 3). In particular, the infections lead to a major induction of monoterpene (camphene, β -myrcene, β -pinene, linalool), sesquiterpene (α -bergamotene) and benzenoid (benzaldehyde, benzothiazole) emissions (Table 3), and the emissions increased linearly with the increasing infection severity (Fig. 6h, i). In contrast, emissions of monoterpenes camphene, β -myrcene, β -pinene and linalool, and sesquiterpene and benzothiazole emissions were below the detection limit in leaves infected severely by *N. anthracinus*. Although benzaldehyde emissions were increased upon infection similarly to LOX products (Table 3), monoterpene emissions even decreased with the degree of infection of *N. anthracinus* (Fig. 4g).

PCA analysis indicated that top two PCA factors contributed 58.9% to the total variation (PC1: 46.1% and PC2: 12.8%) among the emission blends in control leaves and in leaves infected by different wasp species. The distribution of volatile compounds from non-infected and severely infected leaves by different wasp gall species in the PCA loading plot (Fig. 5b) and the distribution of clusters in the PCA score plot (Fig. 5a) showed high quantitative variations in the volatile emission blends among the leaves infected by different gall wasp species. The leaves infected by *Cynips* spp. and *N. albipes* were differentiated from non-infected and *N. anthracinus*-infected leaves by PC1 due to higher monoterpene emissions, whereas the *N. anthracinus* were separated from non-infected leaves in PC2 due to lower isoprene emissions (Fig. 5a). Regarding the volatile compositions of leaves infected by different wasp gall species, isoprene and (*E*)-3-hexenal were differently positioned from the clusters formed by monoterpenes and sesquiterpenes and benzenoids in the plane formed by PC1 and PC2 axes, while other aliphatic derivatives (6-methyl-5-hepten-2-one and 2-ethyl-1-hexanol) were differently positioned relative to terpenoids and benzenoids along PC2 axis (Fig. 5b).

Sources of volatile emissions from infected leaves

Separate analysis of volatile emissions from leaves with galls, leaves with galls removed and galls alone demonstrated that in all cases, leaf tissue is the main source of emitted volatiles (Fig. 6). Only traces of volatiles were detected from the galls in all cases, and the emission rates of intact infected leaves and leaves with the galls removed were not significantly different (Fig. 6).

Discussion

Do *Neuroterus* and *Cynips* galls directly reduce leaf photosynthesis?

Our study highlighted a variety of infection types and severities of infection by gall wasps, whereas clearly different infection types, in particular, the position of galls relative to leaf veins, were observed for different gall wasp species (Fig. 1). According to previous studies, the size of a gall and gall number are related to many factors, for example wasp potential fecundity, frequency of wasps in a given year, leaf position in the crown, position of galls relative to the leaf margin and main veins etc. (Sitch *et al.* 1988; Kato & Hijii 1993; Gilbert *et al.* 1994; Shibata *et al.* 2002; Giertych *et al.* 2013).

We observed that the infection with the species of *Neuroterus* and *Cynips* gall wasps of *Q. robur* leaves resulted in significant reductions in foliage net assimilation rate per leaf area (Fig. 3a-c). We also observed that the decrease in A_n (Fig. 3a-c) was associated with inhibited stomatal conductance in all types of gall infection (Fig. 3d-f), and an overall decrease of leaf water loss in infected plants. However, because photosynthesis decreased more than stomatal conductance, the stomatal closure was associated with a lower leaf water use efficiency (photosynthesis rate per unit transpiration rate, data not shown). It has been reported that galls formed by many species contain chlorophyll and thus, have the potential to photosynthesize (Dorchin *et al.* 2006). Although the photosynthesis never fully compensated for the respiratory costs of galls, it substantially contributed to the maintenance and growth, especially of young galls, reducing their impact as carbon sinks on the host

(Dorchin *et al.* 2006). Thus, for galls appearing green and containing chlorophyll, the photosynthetic performance of gall and healthy leaf tissue can be rather similar and the photosynthesis of the host plant leaves should not necessarily be affected by the gall infection (Oliveira *et al.* 2011; Fernandes *et al.* 2010; Haiden *et al.* 2012). For example, the photosynthesis rate of *Machilus thunbergii* leaves was not significantly affected by the number of cecidomyiid insect galls which have a certain photosynthetic activity (Huang *et al.* 2014). In our study, the color of *Cynips* and *N. albipes* galls was either reddish, yellow or brown, indicating that the gall tissue contained little chlorophyll. However, *N. anthracinus* galls did appear green, and thus, contained chlorophyll, but the net assimilation rate of the host *Q. robur* leaves was reduced more significantly than for *Cynips*- and *N. albipes*-infected leaves. Thus, the reduction in whole leaf photosynthesis occurred despite the galls possibly contributed to leaf photosynthesis. Analogously to our study, the net assimilation rate and stomatal conductance declined in canopy leaves of sugar maple (*Acer saccharum*) trees galled by the maple spindle gall mite *Vasates aceriscrumena* (Patankar *et al.* 2011).

Furthermore, the overall mass fraction and area of galls was relatively small for *Neuroterus* spp. (Fig. 1; Fig. S1; Table 1), suggesting that their direct contribution to leaf photosynthetic activity is small. Although the mass and area of galls of *Cynips* species was much greater, occasionally even exceeding the mass of leaf lamina (Fig. 1; Fig. S1; Table 1), the contents of key mineral elements limiting photosynthesis, N and P, were much lower for the *Cynips* galls than for the leaf lamina (Table 2), suggesting that the physiological activity of the gall tissues was low.

Mechanisms of the quantitative scaling of the reduction of assimilation rate with the severity of infection

Contrary to “green galls”, the reduction of net assimilation rate in the host foliage seems to be a general response to infections by “non-green” parasite galls (Carneiro *et al.* 2014). Given the overall magnitude of reduction of the rate of photosynthesis, occasionally more than two-fold (Fig. 3a-c), this evidence suggests that *Neuroterus* spp. and *Cynips* spp. galls inhibited photosynthesis by a mechanism different from their direct contribution to leaf gas exchange. In fact, the evidence of major photosynthetic reductions suggests that the observed reduction is not simply a “passive response” localized to the sites of infection, but a whole leaf response. However, the mechanism of reduction of photosynthesis is still not entirely clear and both immediate physiological responses as well as longer-term developmental and senescence responses might be involved. Leaf vein pocket galls induced by *Colopha compressa* on leaves of *Ulmus laevis* resulted in photoinhibition of photosynthesis in parallel with decreased photosynthetic electron transport rate and increased non-photochemical quenching (Samsone *et al.* 2012), suggesting that the reduction occurs due to inhibition of primary photosynthetic reactions. Furthermore, in addition to the direct effects on photosynthetic reactions, the larva inside the galls and gall non-photosynthetic tissues could significantly increase whole leaf respiration rate and thereby reduce the rate of net photosynthesis. In our study, the respiration rate was not assessed, but as the reduction in A_n occurred simultaneously with reductions in stomatal conductance (Fig. 3a-c), one might predict that stomatal closure (Fig. 3d-f) was responsible for decreases in net assimilation rate, especially for galls associated with major veins. Indeed, in *N.*

anthracinus galls which were associated with midrib and second order main veins, there was evidence of necrosis of leaf areas downstream the veins colonized by the galls (Fig. 1a). However, for the remaining leaf areas and for all other galling species, the intercellular CO₂ concentration actually increased with increasing the severity of infection (Fig. 3g-i), indicating that a reduction in photosynthetic capacity rather than in stomatal conductance was responsible for the reduction in net assimilation rate. Nevertheless, the presence of non-functional necrotic leaf area in *N. anthracinus* downstream the point of gall attachment does contribute to the reduction of leaf photosynthetic capacity, and there was also evidence of chlorotic and necrotic areas at leaf margins in *N. albipes*- and *Cynips* spp.-infected leaves (Fig. 1a). Yet, the reduction of A_n was up to 2.3-fold for *N. anthracinus*, 1.8-fold *Cynips* spp. (Fig. 3a-c), whereas the necrotic and chlorotic areas were at most ca. 20% for *N. anthracinus* and 5-10% for *N. albipes* and *Cynips* spp., indicating that the spread of necrosis was not the primary cause of reduced A_n in gall-infected leaves.

In addition, galls relying on the carbon and nutrients provided by the host species can importantly modify the sink-source relationships in host leaves, and thus, alter the morphology and nutrient content of leaves during leaf development. In fact, evidence of major reduction in photosynthesis, 25-50% compared with control leaves, induced by eriophyid mite (*Phytoptus cerasicrumena*) in wild cherry (*Prunus serotina*) has been suggested to be indicative of sink competition for carbon and nutrients between developing leaves and growing gall tissue (Larson 1998). In our study, the infections by gall wasps resulted in major decreases in *Q. robur* leaf dry mass per unit area (M_A , Table 2; Fig. 2) for all gall wasp species, and reductions in leaf P content in *N. anthracinus*-infected leaves, and a moderate reduction in leaf N content in *C. quercusfolii*-infected leaves (Table 3). This suggests that the alteration of sink-source relations during leaf growth and gall development is likely. Alternatively, when gall infection occurs in mature leaves, leaves could still undergo a certain structural and chemical readjustment, although the capacity for such modifications is much smaller than in the case of young developing leaves (Naidu & DeLucia 1997; Niinemets *et al.* 2004; Yamashita *et al.* 2002). As A_n is the product of M_A and net assimilation rate per dry mass (A_m), the reduction of M_A in gall-infected leaves provides the major explanation for decreases in A_n in gall-infected leaves. In fact, A_m was even positively correlated with the degree of leaf damage (Table 2), suggesting a certain overcompensation (Niinemets 2016). This overcompensation was associated with increased intercellular CO₂ concentration (Fig. 3g-i) such that despite the reduction in photosynthetic capacity, the photosynthetic machinery operated at a higher CO₂ concentration.

Scaling of constitutive isoprene emissions with the severity of infection

Infection of *Q. robur* leaves by *Neuroterus* and *Cynips* gall wasps resulted in a major inhibition of the capacity of isoprene release (Table 3; Fig. 4a-c). To our knowledge, there have been no reports of the response of isoprene emission to gall infection in plants, but analogous reductions in isoprene emission capacity have been demonstrated in fungal-infected leaves of *Q. robur* (Copolovici *et al.* 2014), *Salix burjatica* x *S. dasyclados* (Toome *et al.* 2010) and *Populus balsamifera* var. *suaveolens* leaves (Jiang *et al.* 2016), and in *Lymantria dispar*-infected leaves of *Quercus robur* (Copolovici *et al.* 2017). These previous studies indicated that the reduction in isoprene emission in fungal-infected leaves was

quantitatively associated with the degree of leaf infection (Copolovici *et al.* 2014; Jiang *et al.* 2016; Toome *et al.* 2010), and an analogous infection severity-dependent scaling of isoprene emissions in gall wasp-infected leaves was also observed in our study (Fig. 4a-c).

The reduction in isoprene emission rate in infected leaves can result from reduced substrate availability for isoprene synthesis and/or decreases in the activity of isoprene synthase, the enzyme responsible for isoprene synthesis. Substrate availability for isoprene synthesis can become limited either by the overall decreases in the activity of the plastidic isoprenoid synthesis pathway (2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway, MEP/DOXP pathway), or due to activation of other plastidic reactions relying on the same substrate pool. Given that a significant fraction of leaf photosynthetic carbon, up to 10% or even more in stressed leaves can be released as isoprene (Sharkey & Yeh 2001), synchronous reductions of isoprene emission and photosynthesis rates have been suggested to reflect limited carbon availability for isoprene biosynthesis due to decreased photosynthesis in fungal-infected leaves (Copolovici *et al.* 2014; Jiang *et al.* 2016). Although the reduction in both the isoprene emission capacity and changes in net assimilation rate occurred simultaneously in gall-infected oak leaves, the reduction in isoprene emission in most severely infected leaves with high M_g/M_l was much larger than the reduction in net assimilation rate (cf. Fig. 3a-c and Fig. 4a-c). As a result, the fraction of leaf photosynthetic carbon going into isoprene emission actually decreased with increasing the degree of leaf infection.

In general, there seems to be a negative relationship between constitutive isoprene emissions and stress-induced emissions of larger isoprenoids such as mono- and sesquiterpenes (Copolovici *et al.* 2014; Jiang *et al.* 2016; Toome *et al.* 2010; Copolovici *et al.* 2017). In particular, both isoprene and monoterpenes are synthesized in plastids via the MEP/DOXP pathway and their synthesis relies on the same plastidic substrate pools of primary intermediates dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP), and these pools are in equilibrium (Rajabi Memari *et al.* 2013; Rosenkranz & Schnitzler 2013). However, the Michaelis-Menten constant (K_m) for DMADP of isoprene synthase is one order of magnitude smaller than that for prenyltransferases that are responsible for condensation of DMADP and IDP to form geranyl diphosphate (GDP), the substrate for monoterpenes (Rajabi Memari *et al.* 2013; Rosenkranz & Schnitzler 2013). This implies that the competition is one-sided and monoterpene emission is favored provided there is a certain monoterpene synthase activity (Rasulov *et al.* 2014).

While this mechanism can well explain the reduction in isoprene emission rate in leaves infected by *N. albipes* and *Cynips* spp. (cf. Fig. 4a-c), monoterpene emissions were at very low level in *N. anthracinus*-infected leaves, and even these low-level emissions actually decreased somewhat with increasing severity of infection (Fig. 4g). This suggests that the reduction in isoprene emission in *N. anthracinus*-infected leaves likely resulted from decreases in isoprene synthase activity, reflecting an overall reduction in leaf physiological activity.

Gall infection severity and induction of lipoxygenase pathway volatiles

All gall wasp infections resulted in an elicitation of the lipoxygenase pathway (LOX) volatiles (Table 3; Fig. 4d-f), also called the green leaf volatiles, that are classic stress compounds typically elicited upon severe stress that leads to membrane-level damage (Copolovici *et al.* 2012; Matsui *et al.* 2012; Scala *et al.* 2013). As in the case of fungal pathogen infection, insect herbivory feeding and methyl jasmonate exposure (Copolovici *et al.* 2014a; Copolovici *et al.* 2014c; Niinemets *et al.* 2013; Jiang *et al.* 2017), LOX emissions increased with the severity of gall infection in all cases (Fig. 4d-f).

Although the emissions were elicited similarly in all gall infection types, the highest emissions were observed in leaves infected by *N. anthracinus* galls that were mainly associated with major veins (Fig. 4d). Such a high LOX emission rate is consistent with previous evidence demonstrating that wounding of major veins results in much greater LOX emissions than wounding of intercostal leaf areas (Portillo-Estrada *et al.* 2015). Such a greater damage is also consistent with visual observations of necrotic leaf areas downstream the site of *N. anthracinus* gall attachment (Fig. 1a).

The question is to what extent the LOX emissions observed in gall-infected leaves reflected a direct damage response from the immediate sites of impact and to what extent they reflected metabolism and derivatization of LOX products diffusing from the sites of infection (Matsui *et al.* 2012). Indeed, contrary to fungal infections and insect herbivory where the emissions are dominated by non-derivatized aldehydes and alcohols such as 3-(Z)-hexenal, 1-hexenol and 1-hexanal (Copolovici *et al.* 2014c; Jiang *et al.* 2016), a large fraction of the LOX blend was due to derivatized products, 6-methyl-5-hepten-2-one and 2-ethyl-1-hexanol, especially in *N. albipes*- and *Cynips* spp.-infected leaves (Table 3). This suggests that a major part of LOX formed in response to direct localized damage was further metabolized in non-damaged leaf parts of *N. albipes*- and *Cynips* spp.-infected leaves. In contrast, the LOX emission blend in *N. anthracinus* had the greatest share of 3-(Z)-hexenal, suggesting that the bulk of LOX were immediately released after damage. Nevertheless, in all cases leaves did continue to emit LOX after gall removal (Fig. 6), suggesting a sustained elicitation response, which might indicate continued spread of leaf necrosis beyond the immediate sites of gall impact.

Contrasting stress-dose dependent terpenoid and benzenoid emission responses upon different gall infections

Previous studies have demonstrated that monoterpene emissions in *Q. robur* leaves are elicited in response to fungal infections (Copolovici *et al.* 2014c) and herbivore infestation (Ghirardo *et al.* 2012; Copolovici *et al.* 2017). However, different biotic stresses elicit different blends of monoterpenes in *Q. robur*. In the case of fungal infection, monoterpene emissions were dominated by α -pinene, limonene, 3-carene, and camphene (Copolovici *et al.* 2014c), while in the case of herbivore infection, the emission were dominated by (*E*)- β -ocimene for *Tortrix viridana* infections (Ghirardo *et al.* 2012) and by α -pinene, ³-carene, limonene and β -phellandrene for *Lymantria dispar* infections, although (*E*)- β -ocimene was a significant component of the emission blend as well (Copolovici *et al.* 2017). In non-infected leaves and in *N. anthracinus*-infected leaves in our study, the low-level emissions

were dominated by α -pinene and limonene (Table 3), but infections by *N. albipes* and *Cynips* spp. led to major increases in β -pinene, camphene and myrcene, whereas the blends also differed among *N. albipes* and *Cynips* spp. infections (Table 3, Fig. 5).

Analogously to LOX products, severe stress often leads to emissions of benzenoids such as methyl salicylate (Beauchamp *et al.* 2005; Copolovici & Niinemets 2016) and benzaldehyde (Copolovici *et al.* 2014c). Similarly to our study, fungal-infection of leaves of *Q. robur* resulted in elicitation of emissions of benzaldehyde, but also methyl salicylate (Copolovici & Niinemets 2016) that was not observed in our study. On the other hand, *N. albipes* and *Cynips* infections resulted in major benzothiazole emissions (Table 3) that have not been observed before in *Q. robur* under different biotic stresses. In other woody plants, benzothiazole has been identified in wounded maple (*Acer negundo*) leaves (Ping *et al.* 2001) and damaged poplar (*Populus simonii* x *P. pyramidalis*) cuttings (Hu *et al.* 2009). Thus, benzenoids further added to the differentiation of the emission blend of oak leaves induced by gall-forming wasps. Altogether these data indicated that different gall-forming wasp species elicited unique VOC blends (Fig. 5), providing diagnostic information that might help discerning different gall infections by the blend of leaf emissions.

Unlike the response to a herbivore attack, in the case of gall infection, elicitation of volatile terpenoids and benzenoids is not always a universal leaf response. It has been reported that galls themselves can also be volatile emitters. For example, the emission of germacrene D, bicyclogermacrene, limonene, and β -pinene were detected in the galls induced by the insect *Baccharopelma dracunculifoliae* (Hemiptera: Psyllidae) on leaves of *Baccharis dracunculifolia* (Damasceno *et al.* 2010). Diverse terpenoid compounds were detected from the galls of gregarious aphid *Slavum wertheimae* on wild pistachio (*Pistacia atlantica*) trees. These terpenoids emitted from the gall acted as olfactory signals and feeding deterrents to enable the gall-inducers to escape being eaten by mammals (Rostás *et al.* 2013). Our analysis of volatile emissions from separated galls and leaf tissue showed that only traces of volatiles were released from the galls in all cases (Fig. 6), indicating that the oak leaf is the primary contributor to the induced volatile emission, and the specialized emissions primarily reflected a plant response.

The induction of monoterpene emission by *N. albipes* and *Cynips* galls together with derivatized LOX and benzenoid emissions in our study can play an important role in indirect defense by gall wasp parasitoids and predators (Shorthouse *et al.* 2005; Tooker & De-Moraes 2008). Indeed, it has been documented that a specialist parasitoid species attacking a *Cynips* gall wasp prefers the odor of gall-infested plants over control plants, although the specific volatile cues involved have not been conclusively identified (Tooker & Hanks 2006). On the other hand, lack of induction or even suppression of monoterpene emission from *Q. robur* by *N. anthracinus* gall infection might imply a strategy employed by galls to reduce the predation risk for the gall inducer and the subsequent herbivore. For example, gelechiid moths were found to suppress the host plant ability to produce volatiles which may help these gall insects to avoid predation or parasitism (Tooker *et al.* 2008). *Heliothis virescens* elicited strong indirect defensive responses in *S. altissima*, but the gall-inducing species and spittlebugs did not (Tooker *et al.* 2008). More significantly, an infestation of *Eurosta solidaginis* appeared to suppress volatile responses to a subsequent attack by a generalist

caterpillar. *Eurosta solidaginis* apparently exerts control over host-plant defense responses that may reduce the predation risk for the gall inducer and any subsequent herbivores, and thus, could influence community-level dynamics, including the distribution of herbivorous insect species associated with *S. altissima* (Besten *et al.* 2015). Similarly, a downregulation of host-plant defense responses by gall-inducing sawflies has been described (Nyman & Julkunen-Tiitto 2000). The way such an inhibition of terpenoid synthesis is achieved is unclear, but in the case of *N. anthracinus* it might reflect the strict association of galls with major veins and be primarily the localized response, mainly reflected in enhanced LOX emissions that rapidly escaped the damaged sites. In contrast, derivatized LOX compounds and major benzenoid emissions might have served as the signals triggering enhanced terpenoid synthesis in *N. albipes* and *Cynips* spp.

Conclusions

This evidence collectively suggests that the quantitative relationships between the severity of infection by *Neuroterus* and *Cynips* galls and foliage photosynthetic capacity mainly reflect modifications in leaf structural characteristics (Fig. 7), and to some extent, chemical characteristics due to alterations in sink-source relationships during leaf development or in mature leaves. Although leaf water use is also reduced in gall-infected leaves, the structural and chemical modifications dominate the changes in photosynthesis, and the intercellular CO₂ concentration actually increased in gall-infected leaves as is often observed upon biotic infections (Fig. 7, Copolovici *et al.* 2014c; Jiang *et al.* 2016). Gall infection had a profound influence on volatile emission by suppressing constitutive isoprene emissions and inducing LOX and benzenoid emissions for infections by all gall wasp species. Yet, major vein-associated *N. anthracinus* galls had a greater LOX emission response than the leaves galled by other species. Furthermore, terpenoid emission responses also seemed to depend on the infection types, with no induction observed for major-vein associated species *N. anthracinus* and large increases in emission of mono- and sesquiterpenes in *N. albipes* and *Cynips* spp. that are primarily associated with intercostal areas. Thus, infections by different gall species result in different strategies employed by the host plant for the defense (Fig. 7). These responses to the infection by different gall-inducing parasites will provide valuable information for diagnosing infections by different gall species using ambient air volatile fingerprints and for predicting the impacts of infections on photosynthetic productivity.

From the perspective of future dispersal of gall infections, it is important to consider that the formation and spread of galls by the parasites including *Neuroterus* and *Cynips* species are regulated by multiply external factors including humidity and temperature. Humid conditions such as moderately cool temperatures and wet conditions supporting high stomatal openness and water and nutrient supply to galls are advantageous for the gall formation. At the northern latitudes, such humid conditions are expected to be increasingly more frequent in the future (Räsänen *et al.* 2004; Kirtman *et al.* 2013). Thus, a highly simplified prediction would be that gall infections are becoming more frequent in future climates in northern latitudes. However, due to the complex life cycles of galling wasps, climate change can also affect winter survival of wasps, lead to a disturbance or loss of potential for sexual reproduction of the gall-inducing parasite. Together, these effects can potentially result in local extinction of the gall-forming species, thereby influencing the

overall geographical distribution and genetic diversity of the gall-forming species. Such complications are illustrated by galling wasp abundance in our study conducted in two years with similar weather conditions. In 2015, there was a mass infection of *N. anthracinus*, but only a minor level of infection was observed in 2016. In contrast, *N. albipes* was not detected in 2015, but it was abundant in 2016, and a *Cynips* spp. were diffusely distributed in both years. Clearly quantitative models of galling wasp influence on tree photosynthesis and volatile emission in future climates need to consider the effects of climate on wasp life cycles.

Supporting Information

Refer to Web version on PubMed Central for supplementary material.

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Summary statement

A huge number of gall wasp species can parasitize oak leaves, but physiological implications of different gall wasps infections are poorly understood. Analysis of effects of four different gall wasp infections on foliage photosynthetic characteristics and volatile emission rates in *Quercus robur* indicated that gall wasp infection resulted in major reductions in foliage photosynthesis rates and elicitation of emissions of green leaf volatiles, mono- and sesquiterpenes and benzenoids in infection severity-dependent manner. Different gall infections resulted in unique emission blends, highlighting a surprisingly selective host volatile response to various gall wasps.

Figure 1.

Representative images of *Quercus robur* upper and lower sides of uninfected leaves (control) and leaves infected by different species of gall-forming wasps (a). Close-ups (b-e) and cross-sections (f-i) of galls of *Neuroterus albipes* (b, c, f, g), *C. quercusfolii* (d, h) and *Cynips divisa* (e, i) formed on leaves of *Quercus robur*. The cross-sections (1 μm thick) were stained with toluidine blue and show the internal structure of the distal part of the galls. The galls of *N. albipes* are typically whitish, but green, pink and red galls are also common (Darlington 1968), and close-ups and cross-sections of both whitish (b, f, larger-celled) and reddish (c, g, smaller-celled) galls are shown. The galls of *N. albipes* are denser and have smaller cells than the galls of *Cynips* species. The blue extrusions outside the galls are wax peels formed at the time of the placement of the sample onto the microscope slide.

Figure 2.

Leaf dry mass per unit leaf area of *Quercus robur* in relation to the severity of leaf infection by gall-forming wasps *N. anthracinus* (a), *N. albipes* (b) and *Cynips* (*C. divisa* and *C. quercusfolii*, c). In this study, the severity of infection is characterized by gall to leaf dry mass ratio (Table 1 for other quantitative measures of the degree of gall infection). Data were fitted by linear regressions ($n = 23$ for all relationships as in Fig. 1) and the corresponding regression equations are: $y = -638x + 82.6$ (a), $y = -632x + 94.2$ (b), and $y = -24.6x + 86.8$ (c).

Figure 3.

Relationships of net assimilation rate (a, b, c), stomatal conductance to water vapor (d, e, f) and intercellular CO₂ concentration (g, h, i) with the severity of leaf infection by *Neuroterus anthracinus*, *N. albipes* and *Cynips* (*C. divisa* and *C. quercusfolii*) gall-forming wasps in *Quercus robur* leaves. The data for net assimilation rate and stomatal conductance were fitted by non-linear hyperbolic regressions, and the data for intercellular CO₂ concentration by linear regressions, except for the net assimilation rate in *Cynips* that was fitted by a linear regression (c). The corresponding regression equations are: $y = 0.938 + 0.0143/(0.00466+x)$ (a); $y = 1.85 + 0.0966/(0.0317+x)$ (b); $y = -1.55x + 4.47$ (c); $y = 67.2 + 0.296/(0.00288 + x)$ (d); $y = 26.6 + 1.70/(0.0158+x)$ (e); $y = 42.7 + 8.99/(0.0926 + x)$ (f); $y = 1864x + 265$ (g); $y = 1581x + 181$ (h); $y = 98x + 184$ (i). $n = 23$ for all regressions.

Figure 4.

Emission rates of isoprene (a-c), lipoyxygenase pathway volatiles (LOX compounds; d-f) and monoterpenes (g-i) of *Quercus robur* leaves in relation to the severity of infection by gall-forming wasps *Neuroterus anthracinus* (a, d, g), *N. albipes* (b, e, h) and *Cynips* species (*C. divisa* and *C. quercusfolii*; c, f, i). The data of isoprene emission in all species were fitted by non-linear regressions ($n = 23$ for all species): $y = 0.862 + 0.00583/(0.00131 + x)$ (a); $y = 0.622 + 0.0857/(0.0196 + x)$ (b); $y = 0.624 + 0.354/(0.0813 + x)$ (c). The data of LOX compounds and monoterpenes were fitted by linear regressions, except for monoterpene emission in *N. anthracinus* (d) that was fitted by a non-linear regression: $y = 10.9x + 0.482$ (d); $y = 0.846x + 0.00473$ (e); $y = 0.0661x + 0.0317$ (f); $y = 0.0347 + 0.000413/(0.00418 + x)$ (g); $y = 23.0x + 0.0862$ (h); $y = 1.036x + 0.371$ (i). $n = 23$ for all relationships.

Figure 5.

Score (a) and loading (b) plot of principal component analysis (PCA) based on the emission rate ($\text{nmol m}^{-2} \text{s}^{-1}$) of the volatiles from the uninfected leaves (2015 and 2016) and leaves infected by *Neuroterus anthracinus*, *Neuroterus albipes* and *Cynips* spp. In the score plot, each symbol represents an individual leaf, either uninfected (2015-yellow triangles and 2016-purple triangles) or infected (black circles: *N. anthracinus*; red diamonds: *N. albipes*; blue squares: *C. quercusfolii*; green squares: *C. divisa*). In the loading plot, the numbers represent different volatiles (the numbers corresponding to the compound numbers in Table

3). Emission rates of compounds positioned closer in the loading plot are more strongly correlated.

Figure 6.

Comparison of average (+SE, $n = 3$) emission rates of isoprene, lipoxigenase pathway volatiles (LOX compounds), monoterpenes and sesquiterpenes between the intact infected leaves (Leaf + gall), leaves with galls removed (Leaf, $n = 3$) and separated galls (Gall, $n = 3$) of *Quercus robur* leaves infected by *Neuroterus anthracinus* (A), *N. albipes* (B) and *Cynips quercusfolii* (C). Averages for different leaf fractions with the same lowercase letter are not significantly different (ANOVA, $P > 0.05$).

Figure 7. Schematic summary of the effects of *Quercus robur* leaf infection by *Neuroterus anthracinus*, *N. albipes* and *Cynips* species on leaf structural and photosynthetic traits and volatile emissions.

Table 1

Gall-forming wasp infection of *Quercus robur* leaves: characteristics of galls and overall degree of infection by different wasp species

Trait	Infecting gall species			
	<i>Neuroterus anthracinus</i>	<i>Neuroterus albipes</i>	<i>Cynips quercusfolii</i>	<i>Cynips divisa</i>
Gall number	6.2 ± 1.0 (69.0)a	4.55 ± 0.44 (43.7)a	1.68 ± 0.19 (60.8)b	1.71 ± 0.29 (44.1)b
Single gall area (cm ²)	0.0698 ± 0.0030 (19.3)a	0.2019 ± 0.0032 (7.0)b	1.18 ± 0.14 (54.5)c	0.99 ± 0.14 (38.6)c
Single gall dry mass (mg)	0.99 ± 0.10 (46.6)a	2.56 ± 0.30 (52.0)b	99 ± 11 (61.0)c	90.59 ± 18.33(53.5)c
Gall dry to fresh mass ratio	0.386 ± 0.006 (6.9)a	0.359 ± 0.030(37.5)a	0.159 ± 0.025 (71.5)b	0.20 ± 0.07 (92.4)b
Gall to leaf dry mass ratio	0.0262 ± 0.0035 (59.9)a	0.040 ± 0.006 (62.7)a	0.61 ± 0.09 (80.6)b	0.41 ± 0.08 (52.9)b
Percentage of leaf area covered by galls (%)	0.89 ± 0.10 (51.3)a	2.16 ± 0.23 (48.1)b	7.6 ± 1.2 (86.2)c	4.03 ± 0.67 (44.2)bc

The data are presented as averages ± SE (CV), where CV is the coefficient of variation ($n = 20$ for *N. anthracinus*, $n = 20$ for *N. albipes*, $n = 13$ for *C. quercusfolii*, $n = 7$ for *C. divisa*). Different letters represent the statistical significance at $P < 0.05$ level according to ANOVA followed by Tukey's tests.

Table 2

Comparison of structural, photosynthetic and chemical traits of uninfected leaves of *Quercus robur* and leaves severely infected by *Neuroterus* (*N. anthracinus* and *N. albipes*) and *Cynips* (*C. quercusfolii* and *C. divisa*) wasps

	Uninfected leaf	<i>Neuroterus anthracinus</i>	<i>Neuroterus albipes</i>	<i>Cynips quercusfolii</i>	<i>Cynips divisa</i>
Leaf dry to fresh mass ratio (g g ⁻¹)	0.508 ± 0.011 (0.579 ± 0.009) ^a	0.45 ± 0.06a	0.433 ± 0.028a	0.560 ± 0.042a	0.64 ± 0.07a
Leaf dry mass per unit leaf area (g m ⁻²)	101 ± 14 (96.5 ± 1.7)	49.0 ± 1.7 *	37.6 ± 9.0 *	53.2 ± 2.4 *	89.1 ± 5.3b
Net assimilation rate per area (μmol m ⁻² s ⁻¹)	5.04 ± 0.20 (3.87 ± 0.33)	1.71 ± 0.18 **	2.84 ± 0.11 *ab	1.86 ± 0.14 **	3.39 ± 0.25 *b
Net assimilation rate per dry mass (nmol g ⁻¹ s ⁻¹)	572 ± 22(468 ± 19)	236 ± 16 **	328 ± 19 *ab	272 ± 13 **	351 ± 18 *b
Stomatal conductance (mmol m ⁻² s ⁻¹)	136 ± 10 (173 ± 13)	83.0 ± 1.9 *	35.9 ± 4.5 **b	56 ± 7 **ab	56.6 ± 1.7 **ab
Interacellular CO ₂ concentration (μmol mol ⁻¹)	168 ± 13 (256.2 ± 3.7)	358 ± 15 **	335.0 ± 5.6 *	341 ± 18 **	250 ± 13 *b
Carbon content (%) ^b	41.6 ± 0.7	42.6 ± 0.8a	42.7 ± 0.5a	41.0 ± 0.9a	
Nitrogen content (%)	2.31 ± 0.16	2.057 ± 0.012a	2.200 ± 0.042a	1.83 ± 0.08b	
Phosphorus content (%)	0.39 ± 0.06	0.173 ± 0.007 *	0.380 ± 0.012b	0.413 ± 0.030b	

^aThe data were measured either in 2015 (data for uninfected leaves in parentheses and corresponding data for *N. anthracinus*) or in 2016 (all other data).

^bIn the case of infected leaves, the galls were removed before estimation of leaf element contents.

In these comparisons, the average (± SE) values of gall to leaf dry mass ratio (M_g/M_l) for infected leaves were 0.052 ± 0.006 for *N. anthracinus*, 0.082 ± 0.006 for *N. albipes*, 1.59 ± 0.05 for *Cynips quercusfolii* and 0.65 ± 0.07 for *Cynips divisa*, indicating a severe infection in each case (Fig. 1). The effect of infection on trait means (comparison of traits for infected vs. corresponding non-infected leaves) was tested by ANOVA ($n = 3$ for both control and severely infected leaves) and the statistical significance is denoted as * - $P < 0.05$, ** - $P < 0.01$. The multiple comparisons among different species were made by Dunnett's tests and different letter represents the statistical significance at $P < 0.05$ level.

Table 3

Emission rates of volatile organic compounds of uninfected leaves of *Quercus robur* and leaves severely infected by *Neuroterus* (*N. anthracinus* and *N. albipes*) and *Cynips* (*C. divisa* and *C. quercusfolii*) wasps

Compounds	Retention index	Emission rate (nmol m ⁻² s ⁻¹)			
		Uninfected leaf	<i>Neuroterus anthracinus</i>	<i>Neuroterus albipes</i>	<i>Cynips</i> (<i>C. divisa</i> and <i>C. quercusfolii</i>)
Aliphatic derivatives					
(E)-3-Hexenal	802	0.0115 ± 0.0012(0.317 ± 0.006) ^a	0.63 ± 0.015 ^a	0.081 ± 0.005 ^{**b}	0.081 ± 0.023 ^{**b}
6-Methyl-5-hepten-2-one	985	0.0263 ± 0.0046(0.0187 ± 0.0033)	0.048 ± 0.006 ^a	0.0382 ± 0.0046a	0.0419 ± 0.0028a
2-Ethyl-1-hexanol	1015	0.0065 ± 0.0015(0.1163 ± 0.0042)	0.173 ± 0.032a	0.023 ± 0.006 ^{**b}	0.0428 ± 0.0037 ^{**b}
Isoprenoids					
Isoprene	507	5.03 ± 0.47(5.3 ± 0.6)	1.45 ± 0.15 ^{**a}	1.18 ± 0.34 ^{**a}	0.69 ± 0.17 ^{**b}
α-Pinene	932	0.0036 ± 0.0007(0.065 ± 0.015)	0.0317 ± 0.0046 ^a	1.20 ± 0.30 ^{**b}	1.44 ± 0.35 ^{**b}
Camphene	948	ND	ND	0.12 ± 0.06a	0.042 ± 0.011b
β-Myrcene	988	ND	ND	0.0131 ± 0.0042a	0.093 ± 0.021b
β-Pinene	980	ND	ND	0.0246 ± 0.0009a	0.071 ± 0.020b
β-OCimene	1046	0.00287 ± 0.00047(0.0152 ± 0.0037)	ND	0.30 ± 0.09 ^{**a}	0.84 ± 0.24 ^{**b}
Limonene	1029	0.00162 ± 0.00022(0.037 ± 0.005)	0.0063 ± 0.0017 ^{**a}	0.153 ± 0.044 ^{**b}	0.248 ± 0.048 ^{**c}
Linalool	1098	ND	ND	0.00099 ± 0.00023a	0.0054 ± 0.0019b
α-Bergamotene	1434	ND	ND	0.020 ± 0.006a	0.018 ± 0.008a
Benzenoids					
Benzaldehyde	967	0.0088 ± 0.0020(0.034 ± 0.007)	0.074 ± 0.019 ^a	0.15 ± 0.07 ^{**b}	0.66 ± 0.10 ^{**c}
Benzothiazole	1218	ND	ND	0.121 ± 0.031a	1.02 ± 0.26b

^a As in Table 2, the data for uninfected leaves in parentheses correspond to control measurements in 2015 when leaves infected with *N. anthracinus* were also measured. All other data correspond to year 2016.

The volatile concentrations were estimated from air samples collected on cartridges filled with carbon-based adsorbents and analyzed with a Shimadzu 2010 Plus GC-MS and thermal desorption system. The limit of detection was 0.5-1 pmol m⁻² s⁻¹ for all studied compounds (see Methods for details).

Data presentation, the degree of infection and statistical analysis as in Table 2. For compounds not detected in the control leaves (ND, emission rate below the limit of detection), no statistical comparisons were made. A C8-C20 hydrocarbon standard (Sigma-Aldrich, St. Louis, MO, USA) was used to obtain the retention indices as in Pazouki *et al.* (2015) and in Jiang *et al.* (2016). Statistical analyses as in Table 2.