



Variation in insect herbivory across an urbanization gradient: The role of abiotic factors and leaf secondary metabolites

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ABSTRACT

Urbanization impacts plant-herbivore interactions, which are crucial for ecosystem functions such as carbon sequestration and nutrient cycling. While some studies have reported reductions in insect herbivory in urban areas (relative to rural or natural forests), this trend is not consistent and the underlying causes for such variation remain unclear. We conducted a continental-scale study on insect herbivory along urbanization gradients for three European tree species: *Quercus robur*, *Tilia cordata*, and *Fraxinus excelsior*, and further investigated their biotic and abiotic correlates to get at mechanisms. To this end, we quantified insect leaf herbivory and foliar secondary metabolites (phenolics, terpenoids, alkaloids) for 176 trees across eight European cities. Additionally, we collected data on microclimate (air temperature) and soil characteristics (pH, carbon, nutrients) to test for abiotic correlates of urbanization effects directly or indirectly (through changes in plant secondary chemistry) linked to herbivory. Our results showed that urbanization was negatively associated with herbivory for *Q. robur* and *F. excelsior*, but not for *T. cordata*. In addition, urbanization was positively associated with secondary metabolite concentrations, but only for *Q. robur*. Urbanization was positively associated with air temperature for

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Q. robur and *F. excelsior*, and negatively with soil nutrients (magnesium) in the case of *F. excelsior*, but these abiotic variables were not associated with herbivory. Contrary to expectations, we found no evidence for indirect effects of abiotic factors via plant defences on herbivory for either *Q. robur* or *F. excelsior*. Additional biotic or abiotic drivers must therefore be accounted for to explain observed urbanization gradients in herbivory and their interspecific variation.

1. Introduction

Urban trees are important for the sustainable development of cities by providing a broad range of ecosystem services within urban areas which often also extend to non-urban matrices (Endreny, 2018; Nowak et al., 2018; Verheyen et al., 2024). For instance, urban trees are excellent filters for fine particles and pollutants such as nitrogen dioxide, sulphur dioxide and ozone (Hamin and Gurran, 2009; Barwise and Kumar, 2020; Diener and Mudu, 2021), and are important carbon sinks that contribute to mitigate effects of urbanization on climate warming (Nowak et al., 2013; Churkina, 2016). They also play a key role in the temperature regulation of cities by buffering so-called urban heat island effects (Gunawardena et al., 2017; Nastran et al., 2019; Wong et al., 2021). In addition, urban trees provide food resources and habitat for associated communities of animals such as insects and birds (Atchison and Rodewald, 2006; Leston and Rodewald, 2006), and contribute to human wellbeing through positive health effects (Endreny, 2018; Gil-lerot et al., 2024).

Urban environments have diverse effects on species interactions that are important for ecosystem functioning. Herbivory in particular, is a predominant interaction that affects urban ecosystem services such as carbon sequestration and soil fertility (Schowalter, 2012; Stam et al., 2014; Turcotte et al., 2014). Studies have shown that trees found at urban sites differ in the magnitude and patterns of herbivory from trees in rural areas, with several biotic (e.g., species diversity, composition) and abiotic (e.g., temperature, light, nutrients) conditions (Johnson and Munshi-South, 2017) being pointed out as responsible for these differences. For instance, some studies have reported increased insect herbivory in urban areas relative to rural habitats (e.g., Cuevas-Reyes et al., 2013; Turrini et al., 2016), whereas others have found inverse patterns (e.g., Bode and Gilbert, 2016; Kozlov et al., 2017; Moreira et al., 2019). Unfortunately, these mixed patterns are difficult to explain as numerous interwinded factors are likely jointly acting to shape herbivory dynamics along urban-to-rural gradients.

Studies often assume and in some cases have offered evidence that factors varying along urbanization gradients directly affect herbivory by causing changes in herbivore diversity or abundance (e.g., physiological or behavioural effects of higher temperatures, artificial light, or lower host plant availability) (Dreistadt et al., 1990; Miles et al., 2019; Theodorou, 2022). However, so far, we know little about the indirect mechanisms (Raupp et al., 2010, 2012). For example, recent studies have shown that urbanization effects on herbivory are mediated by changes in abiotic conditions affecting plant defences including physical (e.g., trichomes, spines) or chemical traits such as secondary metabolites (e.g., phenolics, terpenoids or alkaloids), which in turn could affect herbivore preference or performance (Thompson et al., 2016; Kozlov et al., 2017; Moreira et al., 2019). These abiotically-mediated effects can operate at different scales, from landscape to microhabitat, and may explain a significant portion of urbanization effects on herbivory (Moreira et al., 2019). For example, increased water run-off and soil compaction can cause physiological stress on urban trees, leading to reduced growth. This stress is associated with changes in plant defences, either increases or decreases (the latter via trade-offs), which in turn affect herbivory (Dale and Frank, 2017; Meineke and Frank, 2018; Moreira et al., 2019). Further research is needed to test these indirect effects and tease them apart from direct effects to gain a robust understanding of urbanization on insect herbivory (Moreira et al., 2019).

In this study, we investigated the effects of urbanization on insect

herbivory and its underlying mechanisms for three widespread tree species in western Europe: English oak *Quercus robur* L. (Fagaceae), small-leaved lime *Tilia cordata* Mill. (Malvaceae), and European ash *Fraxinus excelsior* L. (Oleaceae). We collected leaves from 7 to 8 trees of each species along urban-rural gradients across eight European cities (176 trees in total) to estimate insect leaf herbivory and leaf secondary metabolites putatively associated with herbivory. In addition, to address abiotically-mediated effects, we collected microhabitat-level data on microclimatic (air temperature) and soil chemical (pH, carbon, and nutrient content) variables underneath of each tree. We focused our sampling on focal trees that were isolated from other trees or forested areas, often growing in open fields and urban habitats (hereafter solitary trees). The rationale for selecting these solitary trees was that they provide key conditions that buffer other species against abiotic stress while contributing to ecosystem services locally in both agricultural and urban open habitats (Jackson et al., 1990; Manning et al., 2006; Prevedello et al., 2018), and are yet underrepresented in ecological studies. Based on the above, we specifically addressed the following questions: (1) Is the degree of urbanization associated with leaf herbivory and plant secondary metabolite levels? (2) Which are the most important abiotic factors affecting herbivory and plant secondary metabolite levels along the urbanization gradients? (3) Are these abiotic factors associated with herbivory via direct pathways or indirect pathways (through changes in plant secondary chemistry)?

2. Material and Methods

2.1. Study species

We selected *Quercus robur*, *T. cordata*, and *F. excelsior* to investigate urbanization gradients in plant-herbivore interactions across Europe due to their ecological significance, widespread distribution, and contrasting ecological traits. They are long-lived, native deciduous tree species distributed naturally from Spain to Norway (northwards) and from Spain to Russia (eastwards). In natural forests, these tree species support a large community of specialist and generalist insect herbivores, mainly leaf chewers, miners and gall formers (Foggo, 1996; Kozlov et al., 2017; Moreira et al., 2019; Volf et al., 2021). In addition, secondary metabolites such as phenolic compounds, terpenoids, and alkaloids have been studied previously in these species, and are considered putative defensive compounds against herbivores (Kostova and Iossifova, 2007; Moreira and Abdala-Roberts, 2020; Volf et al., 2021).

These species are commonly found in European forests and often planted in urban sites (Kozlov et al., 2017; Moreira et al., 2019), but were expected to differ in their responses to abiotic correlates of urbanization. For instance, *Q. robur* is sensitive to climate extremes, whereas *T. cordata* exhibits moderate drought tolerance and thrives in warm conditions, and *F. excelsior* typically thrives in well-lit environments and prefers moist conditions (San-Miguel-Ayán et al., 2016). We capitalize on this interspecific variation to elucidate the abiotic drivers of variation in urbanization effects on plant defences and herbivory, ultimately informing on urban forest management aimed at promoting forest health and associated ecosystem services (e.g., biodiversity conservation).

2.2. Study sites and tree selection

We selected eight European cities (“sites” hereafter) varying in

population size from 82,000 to 1,500,000 inhabitants and spanning 14° in latitude (49°N to 63°N) across five countries (Fig. 1A). Across sites, trees and herbivores experience more than a doubling in mean annual precipitation (619–1123 mm) and an increase of 6.6 °C in mean annual temperature (4.4 °C to 11.0 °C) (information between 1991 and 2021: <https://en.climate-data.org/>), covering most (>80%) of the latitudinal and climatic gradients experienced by these species throughout their distribution ranges (San-Miguel-Ayanz et al., 2016).

At each site, we selected 7–8 trees of each species (176 trees in total) along a 10–30 km transect starting at the city centre and ending in rural landscape with very little to no urbanization (i.e., a continuous urban-to-rural gradient) (Fig. 1B). Trees were selected with a spacing of about 1–2 km (Fig. 1B). All selected trees grew alone in a 10-m radius, were mostly single-stemmed (with a diameter at the breast height >50 cm) and had no visual signs of recent (<1–2 years) heavy pruning. Moreover, the ground around the trees was not paved, but rather were lawns and grasslands. For each tree, we estimated the fraction of built-up area in a 75-m radius around the individual based on 2021 ESA WorldCover maps (10 m resolution; <https://worldcover2021.esa.int/>). Built-up area was defined as land covered by buildings (both residential and industrial),

roads, other man-made structures impermeable to water and is considered a proxy of the degree of urbanization (De Pauw et al., 2024). We selected a 75-m radius because the magnitude of abiotic effects has been found to be largest at local scales of a 60–90 m radius around individual trees (Ziter et al., 2019).

2.3. Field sampling and herbivory measurements

Toward the end of the growing season, in July–August 2022, we randomly selected two branches from each tree, approximately 4 m from the ground. From each branch, we randomly collected ten fully expanded leaves of roughly the same age (based on their position along the branch, south side of the tree, crown exterior area), colour, and consistency. These leaves were pooled into a single sample to assess herbivory and leaf secondary metabolites. Our leaf sampling campaign was designed to minimize phenological differences in herbivory and leaf secondary metabolites across sites. In addition, this sampling provided a rough assessment of damage occurring later in the season (as these species produce several flushes) and cumulative herbivory over the growing season (as leaves remain on plants for most of the growing season). Most of the herbivore damage observed on sampled leaves were due to chewing insects and to a lesser extent (<3% of cases) by leaf miners or other herbivore guilds. We photographed all leaves with a Samsung Galaxy A30s (25 effective megapixels, 4 × digital zoom) and estimated the percentage of leaf area consumed by chewing insects (“insect herbivory” hereafter) using BioLeaf - Foliar Analysis™ (Brandoli Machado et al., 2016). We used the average value across leaves for statistical analyses. Overall levels of insect leaf damage were relatively low (<5%; see Results), but fell within the typical range for these species (e.g., Moreira et al., 2019; Moreira et al., 2024), and over several growing seasons have been shown to impose significant detrimental effects in temperate tree species, including reductions in branch growth and seed and biomass production (Kozlov et al., 2017).

2.4. Quantification of leaf secondary metabolites

We selected eight leaves (out of the 20 originally sampled) per tree with little or no herbivore damage and oven-dried them at 40 °C for 48 h to quantify secondary metabolites. Our aim by selecting undamaged leaves was to avoid variation in secondary metabolites due to local induction (Abdala-Roberts et al., 2016), and obtain a rough estimate of constitutive chemical defences responding to long-term patterns of herbivore pressure and abiotic forcing (Abdala-Roberts et al., 2016).

We extracted leaf secondary metabolites from 20 mg of dry pulverized leaf tissue with 1 mL of 70% aqueous methanol in an ultrasonic bath for 15 min, followed by centrifugation (Moreira et al., 2024). We then transferred the extracts to chromatographic vials and performed untargeted analysis of leaf secondary metabolites using ultra-high performance liquid chromatography-high resolution tandem mass spectrometry (UHPLC-HRMS/MS). Specifically, we employed an Acquity UPLC I-Class (Waters Corporation, Milford, MA, USA) coupled to a Synapt XS QTOF (Waters). The system was controlled by Masslynx 4.2. Chromatographic separation was done on an Acquity UPLC HSS T3 column (100 × 2.1 mm, 1.8 μm, Waters) at a flow rate of 0.5 mL/min. The mobile phases were milliQ water (phase A) and acetonitrile (phase B), both supplemented with 0.05% formic acid. The gradient started at 2% B, increased linearly to 43% B in 5.4 min, then to 100% B in 0.4 min, hold at 100% B for 2.0 min, and back to 2% B for 3.0 min. The column was maintained at 27 °C. The injection volume was 0.25 μL. The flow was directed to the mass spectrometer only between 1.3 min and 5.5 min. QTOF data were acquired in negative electrospray ionization in the data-independent acquisition mode (i.e., MSE with low collision energy of 4 eV and high collision energy ramp of 10–60 eV, scan time 0.2 s each). The source parameters were capillary voltage –1.0 kV, source temperature 140 °C, cone voltage –25 V, cone gas flow 250 L/h, desolvation temperature 500 °C, desolvation gas flow 1000 L/h. A 0.1

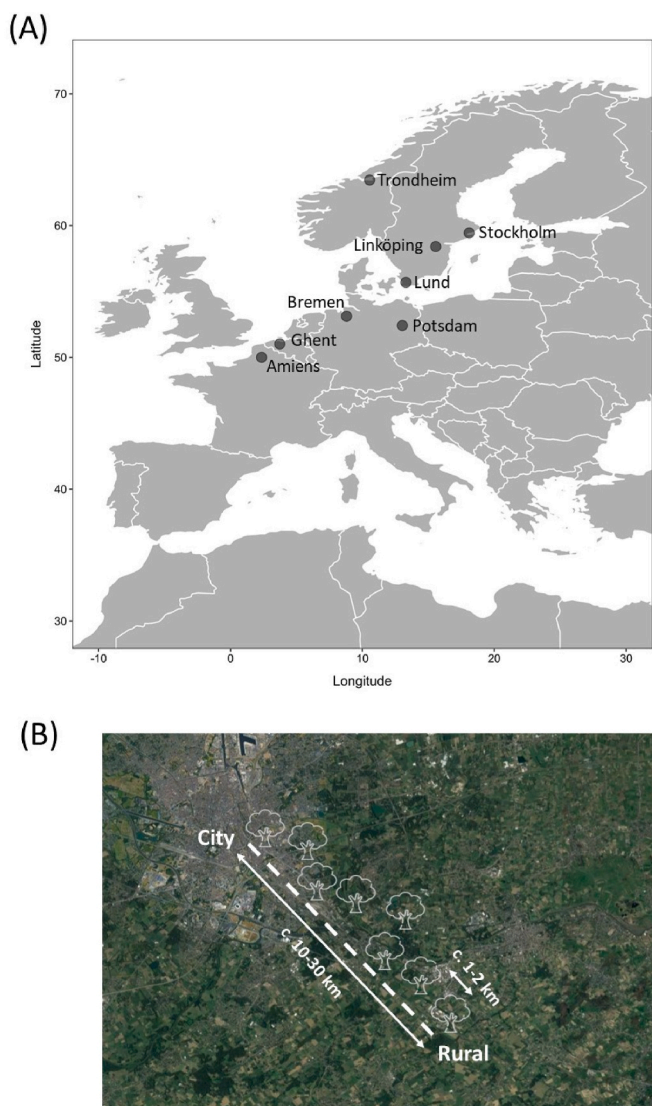


Fig. 1. (A) Map showing the spatial layout of eight European cities where we sampled focal trees. (B) Diagram showing trees of each species selected along a 10–30 km transect, starting in a city and extending into rural areas with little urbanization. Trees were selected with a spacing of about 1–2 km.

µg/mL solution of leucine-enkephalin was infused as lockmass through the LockSpray probe with a frequency of 30 s. The profile data were then lockmass corrected and centroided in Masslynx 4.2, converted to MzML using MSConvert (Adusumilli and Mallick, 2017). We then used MS-DIAL 4.9 software for peak detection (Tsugawa et al., 2015), deconvolution and assignment of parent masses to each of the spectra fragmented from the DIA data (noise threshold MS1: 5000 & MS2: 100). Peak table and spectral data exported from MS-DIAL were annotated using SIRIUS 5.8.6 software (Dührkop et al., 2019), including CSI:FingerID to identify structure and the CANOPUS tool to predict compound classes, super classes and pathways. Structural classification at the class level provided by NPClassifier was used for statistical analysis (Kim et al., 2021). Our untargeted analysis using UHPLC-HRMS/MS is designed to detect non-volatile secondary metabolites. We identified 59 groups of secondary metabolites (Table S1 in the Supplementary material).

2.5. Measurement of microhabitat-related climatic and soil variables

For each tree, we estimated the microclimate-level air temperature (°C) under the canopy by attaching climatic-data loggers (Lascar-EL-USB2, Lascar Electronics, Erie, PA, USA; range of -35 to +80 °C, accuracy of 0.45 °C) to each tree at a height of 3 m from the ground. Loggers were consistently attached at the north side of the tree trunk to exclude variation due to differences in orientation and to limit exposure to direct sunlight. We protected loggers from direct sunlight using white polyvinyl chloride radiation shields (Maclean and Klinges, 2021). Temperature measurements were performed every 30 min for 12 months (end of August 2022 to early September 2023). For statistical analyses, we used mean daily temperature from May to August 2023 as a general measure of climatic conditions present during the growing season. Previous work at the study sites has shown that growing season microclimatic conditions remain highly consistent across years (Fig. S1 in the Supplementary Material), therefore justifying the use of 2023 data as an approximation to 2022 data which is when leaf sampling took place but data loggers were not yet available to record microclimatic conditions.

We also collected soil samples to quantify soil characteristics, namely: pH, carbon concentration, and the concentration of five macro-elements (Ca, K, Mg, N, P). Previous work has demonstrated that urbanization can lead to increased soil acidity and fertilization, primarily due to atmospheric pollution and reduced carbon content due to soil carbon being released into the atmosphere as CO₂ through soil disturbance and microbial decomposition (Marcotullio et al., 2008). To this end, immediately after leaf collection, we collected six soil subsamples at a depth of 0–10 cm around each tree (at a maximum distance of 250 cm from the stem of the focal tree, mostly within the limits of the tree canopy projection) which were pooled into a single sample for chemical analyses. We dried all samples to a constant weight at 40 °C and for at least 48 h, then ground and sieved them over a 2 mm mesh. Then, we estimated pH-H₂O by shaking a 1:5 ratio soil/H₂O mixture for 5 min at 300 rpm and measuring with an Orion 920A pH meter with a Ross sure-flow 8172 BNWP pH electrode model (Thermo Scientific Orion, USA). We determined carbon and nitrogen content by sample combustion at 1150 °C and gas measurements by a thermal conductivity detector in a CNS elemental analyser (vario Macro Cube, Elementar, Germany). We measured total Ca, K and Mg by inductively coupled plasma optical emission spectroscopy (Thermo Scientific™ iCAP™ 7400 ICP-OES) after complete destruction of the soil samples with HClO₄ (65%), HNO₃ (70%) and H₂SO₄ (98%) in Teflon bombs for 4 h at 150 °C. Finally, we determined Olsen phosphorus which is considered as the available P for plants within one growing season (Gilbert et al., 2009) by extraction in NaHCO₃ (POlsen; according to ISO 11263:1994(E)) and colorimetric measurement according to the malachite green procedure (Lajtha et al., 1999).

2.6. Statistical analyses

Tree species variation in herbivore damage – We first conducted a linear model analysis to test the effects of tree species (three levels: *Q. robur*, *F. excelsior*, *T. cordata*), site (eight levels: Amiens, Bremen, Ghent, Linköping, Lund, Potsdam, Stockholm, Trondheim), and their interaction (all fixed factors) on herbivory. We log-transformed herbivory data to achieve normality of residuals. We implemented these models using the *lm* function from the *stats* package in R ver. 4.3.3 (R Core Team, 2024).

Associations between degree of urbanization and leaf herbivory or secondary metabolites – For each tree species, we conducted a linear mixed model (LMM) analysis to test the associations between the degree of urbanization (i.e., the fraction of built-up area around the trees; fixed factor) and herbivory or leaf secondary metabolites, using data at the individual tree level. Models also included site as a random factor. We log-transformed herbivory data to achieve normality of residuals. Rather than testing for associations between the degree of urbanization and each type of secondary metabolite, for each tree species we summarized variation in secondary chemistry (59 compound groups, see above) using a principal component analysis (PCA). We then used the z-values from the first two principal components (PCs) to test for the associations between urbanization degree and metabolites. We implemented these LMMs using the *lmer* function from the *lmerTest* package (Kuznetsova et al., 2017) in R ver. 4.3.3 (R Core Team, 2024). We performed the PCA analyses using the *PCA* function from the *FactoMineR* package (Lê et al., 2008) in R (R Core Team, 2024).

Abiotic correlates of urbanization underlying herbivory – Whenever the degree of urbanization was significantly associated with herbivory or leaf secondary metabolites in the above models, we proceeded to test abiotically-mediated mechanisms underlying such patterns following a two-step process. First, we tested for associations between abiotic factors and the degree of urbanization to identify the most important abiotic correlates of urbanization. To do so, for each species, we conducted individual LMMs testing for an effect of the degree of urbanization on air temperature and on z-values from the first two PCs of a PCA analyses of soil variables (as we did for leaf metabolites). These models included site as a random effect.

Second, abiotic factors significantly associated with the degree of urbanization from these models were used in a piece-wise structural equation model (PSEM) to investigate indirect associations between abiotic factors and herbivory through secondary metabolites (Lefcheck, 2016). For each species, we tested for (1) direct associations between the selected abiotic factors and secondary metabolite PCs (see above), and between secondary metabolite PCs and herbivory, as well as (2) the direct and indirect associations (i.e., through secondary metabolites) between abiotic factors and herbivory. We used leaf secondary metabolites as a predictor of herbivory rather than the inverse, as preliminary analyses indicated that these traits were negatively associated with leaf damage, i.e., compounds negatively predicted leaf damage suggesting they confer biotic resistance. The PSEM included site as a random factor. We estimated PSEM direct associations as standardized path coefficients, whereas indirect associations were calculated by multiplying the specified path coefficients for direct relationships constituting the indirect pathway. We assessed the goodness of fit of the overall model with a ‘test of direct separation’ based on the Fisher’s C-test (Lefcheck, 2016). We implemented the PSEM and obtained direct effect coefficients (i.e., standardized path coefficients) using the *psem* function of the *piecewiseSEM* package (Lefcheck, 2016), and obtained bootstrapped indirect effects (i.e., multiplied path coefficients) and associated 95% confidence intervals using the *semEff* function from the *semEff* package (Torchiano, 2020) in R ver. 4.3.3 (R Core Team, 2024).

3. Results

3.1. Variation patterns in herbivore damage among tree species

Insect leaf herbivory significantly varied (up to 9.7-fold) among tree species ($F_{2,152} = 40.21$, $P < 0.001$; *Q. robur*, mean \pm SE: 2.21 ± 0.39 %, range: 0–9.90 %; *F. excelsior*, mean \pm SE: 4.46 ± 0.44 %, range: 0–27.07 %; *T. cordata*, mean \pm SE: 0.47 ± 0.37 %, range: 0–6.08 %) (Fig. 2). Neither the site ($F_{7,152} = 1.39$, $P = 0.214$) nor the interaction between tree species and site ($F_{14,152} = 1.49$, $P = 0.119$) significantly affected herbivory (Fig. 2).

3.2. Principal component analyses of leaf secondary metabolites and soil chemical variables

For *Q. robur*, two axes explained 39% of the variance across groups of leaf secondary metabolites. The first principal component (“PC1 metabolites”) explained 32% of the variance and was positively related to the concentration of flavonoids, phenolic acids, and tyrosine alkaloids (Fig. 3). The second principal component (“PC2 metabolites”) explained 7% of the variance and was positively related to the concentration of proline alkaloids, mycosporine derivatives and fatty acids and conjugates (Fig. 3). For *Fraxinus excelsior*, two axes explained 59% of the variance across groups of leaf secondary metabolites. The first principal component (“PC1 metabolites”) explained 51% of the variance and was positively related to the concentration of phenolic acids, phenylethanoids, and nucleosides (Fig. 3). The second principal component (“PC2 metabolites”) explained 8% of the variance and was positively related to the concentration of fatty acids and conjugates, fatty esters, and chromanes (Fig. 3). For *Tilia cordata*, two axes explained 32% of the variance across groups of leaf secondary metabolites. The first principal component (“PC1 metabolites”) explained 19% of the variance and was positively related to the concentration of flavonoids, phenolic acid, and lignans (Fig. 3). The second principal component (“PC2 metabolites”) explained 13% of the variance and was negatively related to the concentration of meroterpenoids, oligopeptides, and fatty esters (Fig. 3).

In addition, two axes explained most variance (53% for *Quercus robur*, 56% for *Fraxinus excelsior*, and 63% for *Tilia cordata*) across soil

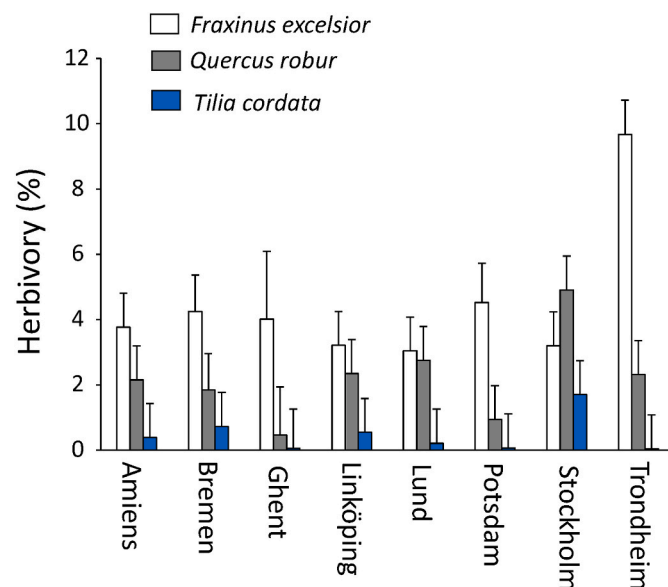


Fig. 2. Leaf herbivory (estimated as the percentage of leaf area consumed by chewing insects) on *Quercus robur*, *Fraxinus excelsior*, and *Tilia cordata* trees sampled along a rural-to-urban gradient in eight European cities (Amiens, Bremen, Ghent, Linköping, Lund, Potsdam, Stockholm and Trondheim). Values are model least-square means (and SE).

chemical variables (Fig. 4). The first principal component (“PC1 soil”) was positively related to the concentration of N and C, whereas the second principal component (“PC2 soil”) was positively related to the concentration of Mg (Fig. 4).

3.3. Associations between the degree of urbanization and leaf herbivory or secondary metabolites

We found a significant negative association between the degree of urbanization and herbivory for *Q. robur* and *F. excelsior*, but not for *T. cordata* (Fig. 5A–C). In addition, there was a significant positive association between the degree of urbanization and PC1 metabolites (positively related to the concentration of flavonoids, phenolic acids, and tyrosine alkaloids; Fig. 3) for *Q. robur*, but not for *F. excelsior* or *T. cordata* (Fig. 5D–F). There was no significant association between the degree of urbanization and PC2 metabolites for any tree species (Fig. 5G–I).

3.4. Abiotic correlates of urbanization effects on herbivory

For *Q. robur*, one of the two species exhibiting a significant gradient in herbivory associated with the degree of urbanization, the LMMs indicated a significant positive association between the degree of urbanization and air temperature ($t = 3.01$; $P = 0.004$), but not between urbanization and soil PCs (PC1 soil: $t = -0.54$; $P = 0.592$; PC2 soil: $t = 0.98$; $P = 0.332$). Accordingly, we conducted a follow-up PSEM including only air temperature and its direct and indirect associations with herbivory and secondary metabolites. Results showed no significant direct associations between temperature and herbivory or secondary metabolites (Fig. 6A). In addition, and despite a significant negative association between herbivory and PC1 metabolites (axis positively related to the concentration of flavonoids, phenolic acids, and tyrosine alkaloids; Fig. 3), we found no significant indirect association between temperature and herbivory via secondary metabolites (Fig. 6A).

Likewise, for *F. excelsior*, LMMs also indicated a significant positive association between the degree of urbanization and air temperature ($t = 5.07$; $P < 0.001$), as well as a negative association with PC2 soil (positively related to magnesium concentration; Fig. 4) ($t = -2.34$; $P = 0.024$), but not with PC1 soil ($t = -1.17$; $P = 0.251$). Subsequently, we conducted a PSEM testing for direct and indirect associations between temperature, PC2 soil, and herbivory. Results from this model showed no significant direct associations between herbivory and any of the abiotic factors (Fig. 6B). In addition, and despite positive associations between temperature and PC1 metabolites (positively related to the concentration of phenolic acids, phenylethanoids, and nucleosides; Fig. 3) and PC2 metabolites (fatty acids and conjugates, fatty esters, and chromanes; Fig. 3) (Fig. 6B), there were no significant associations between secondary metabolites and herbivory (Fig. 6B), as well as no indirect associations between either abiotic factor and herbivory (i.e., through secondary metabolites) (Fig. 6B).

4. Discussion

Degree of urbanization correlated negatively with insect herbivory for two species, namely *Q. robur* and *F. excelsior*. In contrast, no detectable association was found for *T. cordata*, which exhibited negligible levels of leaf damage (0.44%). Analyses also indicated a positive association between degree of urbanization and leaf secondary metabolite concentrations (PC1 defences), but only for *Q. robur*. Follow-up tests including abiotic factors showed that air temperature was positively associated with degree of urbanization for *Q. robur* and *F. excelsior*, and in the case of *F. excelsior*, degree of urbanization was also negatively associated with soils with high levels of magnesium (PC2 soil). Counter to expectations, however, these abiotic proxies of urbanization degree extent were not directly or indirectly associated with herbivory for either of these species. Indirect linkages did not emerge either because

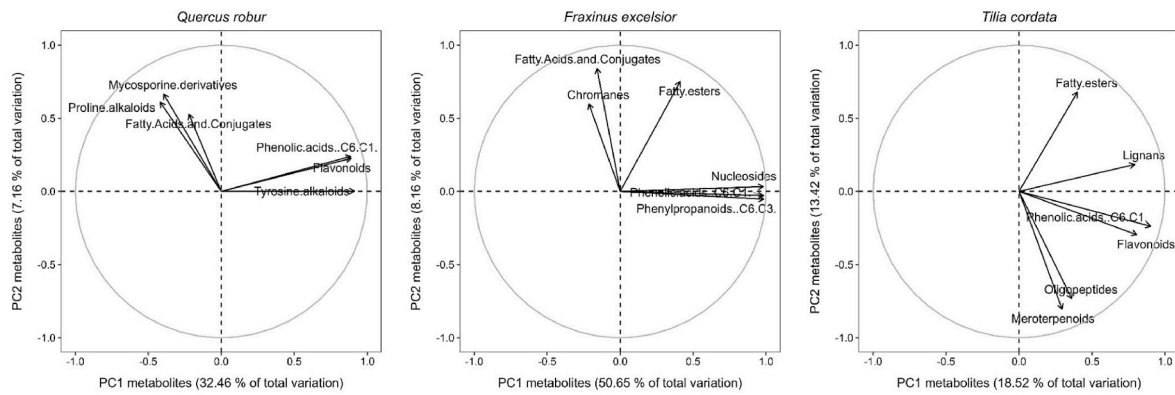


Fig. 3. Ordination using a principal component analysis of leaf secondary metabolites (59 compound groups) in *Quercus robur*, *Fraxinus excelsior*, and *Tilia cordata* trees sampled along a rural-to-urban gradient in eight European cities.

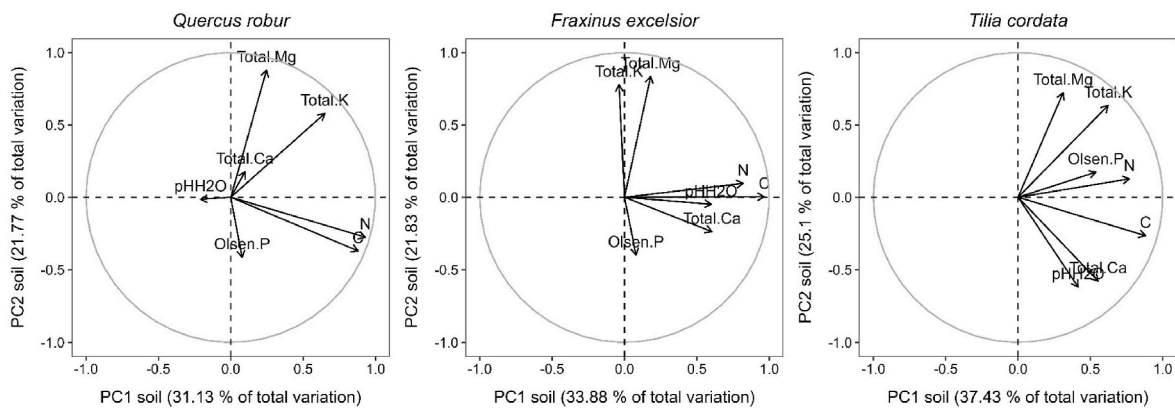


Fig. 4. Ordination using a principal component analysis of seven soil chemical variables in *Quercus robur*, *Fraxinus excelsior*, and *Tilia cordata* trees sampled along a rural-to-urban gradient in eight European cities.

abiotic variables were not associated with defences (*Q. robur*, despite herbivory being associated with defences), or because herbivory was not associated with defences (*F. excelsior*, despite temperature being associated with defences). As a whole, evidence gathered from the present study adds cross-species, continental-scale evidence that urbanization reduces herbivory, while rejecting the idea of abiotically-mediated bottom-up controls as a predominant force behind these herbivory gradients.

The negative association between degree of urbanization and insect leaf herbivory for *Q. robur* and *F. excelsior* counters past work posing that herbivory should be higher in urban ecosystems due to factors limiting plant defensive investment and/or weakening top-down trophic control by natural enemies (reviewed by Dreistadt et al., 1990; Raupp et al., 2010). Instead, our findings agree with recent studies, also on herbivory by leaf-chewing insects, which have consistently found lower damage in urban (vs. rural) locations or increasing degree of urbanization (e.g., Bode and Gilbert, 2016; Kozlov et al., 2017; Moreira et al., 2019; Fenoglio et al., 2020). Within this latter cluster of studies, our results are consistent with a study by Moreira et al. (2019) reporting a 30% reduction in leaf chewer damage on *Q. robur* for urban relative to rural sites across 18 European cities. Similarly, Kozlov et al. (2017) found that herbivory was, on average, 16.5% lower in urban than in rural habitats in Europe across 11 tree species, including *Q. robur* and *T. cordata*, with patterns being contingent on city size. One explanation for these patterns could be that urbanization decreases insect abundance and diversity due to processes such as habitat loss, reduced host plant density, pollution, or changes in microclimates, which in turn leads to lower herbivory levels (Raupp et al., 2010). For instance, since an insect's complete life cycle may not necessarily occur on a single tree, low tree

density in more urbanized areas can hinder the movement and dispersal of insects between trees, potentially exacerbating the negative effects of urbanization on herbivory (Schmitt and Burghardt, 2021). In addition, some studies have found increases in predator (e.g. insectivorous birds) density as a response to urbanization, primarily attributed to enhanced food availability, and presumably contributing to reduce herbivore pressure (Marzluff, 2001; Kozlov et al., 2017; Valdés-Correcher et al., 2022). Lastly, it is also conceivable that herbivores do not find sufficiently favourable conditions in urban areas for their complete or optimal development, for example because the host plants necessary for egg laying (or development of subsequent stages) are rare and/or sparsely distributed across urban landscapes (Raupp et al., 2010). In speculating about these possible causes, it is important to keep in mind that while *Q. robur* and *F. excelsior* exhibited the same pattern, the underlying factor or factor combinations might differ, warranting further investigation. This might include, for example, differences in insect herbivore composition or abundance associated to each tree species, whereby differences in insect dispersal or diet breadth could shape different responses to host availability or susceptibility to predation.

We also found that the degree of urbanization was positively associated with the amount of leaf secondary metabolites in *Q. robur* (PC1 defences, i.e., phenolics and alkaloids), suggesting that that oak trees are more highly defended in or close to urbanized areas. Urban environments often have nutrient-poor soils, limited water availability, contaminants or other resource-acquisition constraints, combined with higher temperatures, which can jointly or individually drive physiological stress on plants (Barbosa, 2020). One plausible scenario is that such stressors lead to slower growth which can in turn result in greater allocation to defences (e.g., via growth-defence trade-offs; Coley et al.,

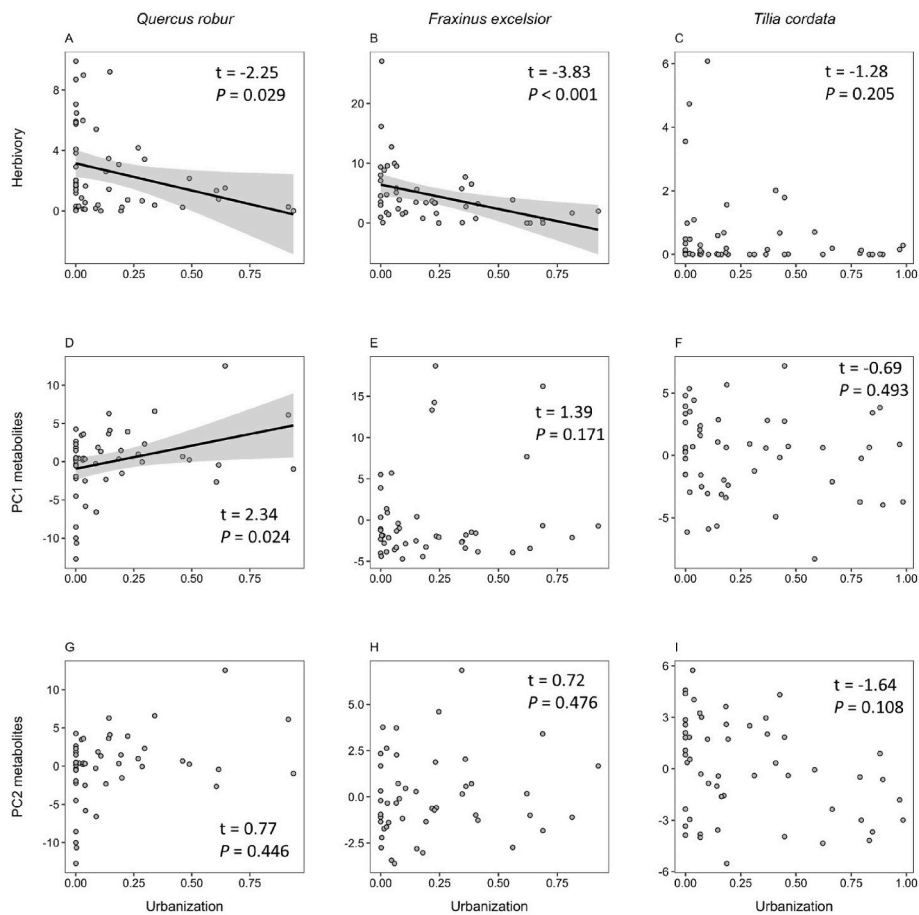


Fig. 5. Associations between the degree of urbanization (estimated as the fraction of built-up area in a 75-m radius around the trees) and leaf herbivory (estimated as the percentage of leaf area consumed by chewing insects) or leaf secondary metabolite concentration (PC1 metabolites and PC2 metabolites, see *Methods*) on (A, D, G) *Quercus robur*, (B, E, H) *Fraxinus excelsior* and (C, F, I) *Tilia cordata* trees sampled along a rural-to-urban gradient in eight European cities. Student's t-test (*t*) and *P*-values are shown. Circles represent individual trees (*N* = 59 for *Q. robur*, *N* = 55 for *F. excelsior*, *N* = 62 for *T. cordata*). Black solid lines represent significant (*P* < 0.05) relationships.

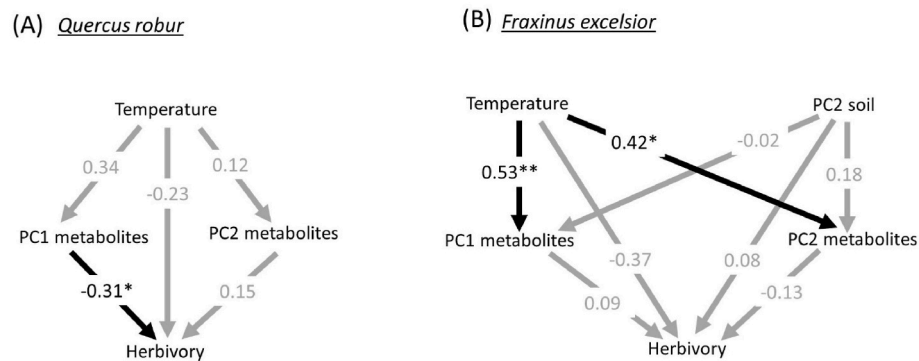


Fig. 6. Diagram showing results from a piece-wise structural equation model (PSEM) testing for direct and indirect associations between abiotic correlates of the degree of urbanization (temperature, soil variables), leaf secondary metabolites (PC1 metabolites and PC2 metabolites), and leaf herbivory on (A) *Quercus robur* and (B) *Fraxinus excelsior* adult trees sampled along a rural-to-urban gradient in eight European cities. Significant (**P* < 0.05, ***P* < 0.01) and non-significant path coefficients (arrows) are in black and grey, respectively. Indirect associations were not significant and are therefore not shown for ease of visualization. Explained variance in panel A: herbivory = 0.19; PC1 metabolites = 0.50, PC2 metabolites = 0.30. Fisher's *C* = 2.20, *P* = 0.333 (model AICc = 767.37). Explained variance in panel B: herbivory = 0.22; PC1 metabolites = 0.71, PC2 metabolites = 0.23. Fisher's *C* = 3.40, *P* = 0.183 (model AICc = 761.51).

1985), even if herbivory is lower since fitness costs to tissue loss to herbivory increase. Notably, a previous study by Moreira et al. (2019) also on *Q. robur* instead found the opposite, i.e., trees in urban locations had lower levels of leaf chemical defences (condensed and hydrolysable tannins) compared to those found in rural areas. Differences in sampling

design between studies could explain this difference, particularly the dichotomous sampling design based on a comparison of rural versus urban sites in Moreira et al. (2019) compared to our approach of testing for urbanization gradients. The latter approach might better capture variation in herbivory and leaf traits to the extent that landscape-level

features make variation naturally fall along a continuum. That said, *F. excelsior* and *T. cordata* did not exhibit urbanization clines in leaf defences, suggesting alternative or additional mechanisms at work (see discussion ahead). Further work is needed to understand these species-specific responses to elucidate the mechanisms by which urbanization shapes plant defences and its links to plant physiology and associated insect communities.

Abiotically-related controls of urbanization effects on species interactions are presumably important but have been seldom explicitly tested (see review by Theodorou, 2022). We found that air temperature increased with degree of urbanization (for both *Q. robur* and *F. excelsior*) whereas soil nutrient conditions such as the amount of magnesium decreased with urbanization (for *F. excelsior*). High temperatures characteristic of urban heat islands, for example, can influence insect behaviour, physiology, and life cycles, leading to lower insect population sizes or reduced feeding activity (Deutsch et al., 2008; Raupp et al., 2010; Schmitt and Burghardt, 2021). That said, neither temperature nor soil features were associated with herbivory, rejecting the idea of climatic- and soil-related direct effects on herbivory along the studied urbanization gradients. Moreover, there was no evidence for indirect associations between abiotic correlates of urbanization and herbivory. In the case of *Q. robur*, these effects were absent because herbivory was associated with defences, but temperature was not. Similar to these results, Moreira et al. (2019) found no evidence for indirect associations between urbanization and herbivory in *Q. robur* through CO₂ emissions-related changes in leaf chemical defences. It is worth noting, however, that there was an urbanization gradient in defences for this species, based on which that indirect links to herbivory involving other unmeasured abiotic factors cannot be ruled out. For *F. excelsior*, on the other hand, temperature was associated with defences, but herbivory was not associated with defences, and, more importantly, there was no urbanization effect on defences to start with. Further research should include a more comprehensive analysis of abiotic factors influencing herbivory in urban environments. For instance, drought, pollution, changes in light, and moisture levels are critical factors that can significantly influence the effects of urbanization on herbivory dynamics. In particular, urban environments often lead to fragmented habitats and altered microclimatic conditions, which exacerbate water limitation and stress for both plants and insects (Fenoglio et al., 2020). Consequently, herbivorous insects experience stunted growth, decreased reproductive success, and increased mortality rates due to the diminished quality and quantity of their food plants (Fenoglio et al., 2020). In addition, pollutants and contaminants in the soil and water, can directly impact plant health and defensive chemistry, potentially altering their susceptibility to herbivores (e.g., Katoh et al., 1989). Changes in light availability due to urban structures and artificial lighting can also affect plant chemistry, phenology, and growth rates, with this in turn affecting herbivore abundance and behaviour (e.g., Cieraad et al., 2023). Finally, changes in moisture levels, often exacerbated by urbanization effects on hydrology and soil compaction, can affect plant water stress tolerance and nutrient availability, which in turn may influence herbivore abundance and feeding behaviours (e.g., Kuczyk et al., 2021). Understanding the individual and joint effect of these factors is crucial for managing pest control in urban forests.

In summary, our findings challenge the view that urban ecosystems should experience higher herbivory and that abiotic bottom-up forcing shapes these patterns. The positive association between urbanization and leaf secondary metabolites in *Q. robur*, but not in the other species, warrants further investigation, as well as drivers of inter-specific variation in these associations. Unmeasured abiotic factors or other types of tree defensive traits may play a role and deserve further attention. Overall, our study contributes to understanding the ecological mechanisms by which urbanization affects plant-herbivore interactions and explain tree inter-specific variation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2024.109056>.

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