SCUOLA DI DOTTORATO

UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Dipartimento di / Department of

Biotecnologie e Bioscienze

Dottorato di Ricerca in / PhD program

Tecnologie convergenti per i sistemi biomolecolari (TeCSBi)

Ciclo / Cycle

XXXVI

Pollination Ecology in Human-Altered Landscapes: Exploring Nutritional Dynamics and Implications for Food Security

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Emiliano Pioltelli

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Anno Accademico 2022-2023

Abstract

In the contemporary pursuit of sustainable development, understanding the relationship among ecosystems, biodiversity, and human well-being is crucial, especially in urban and periurban contexts. Biodiversity is the foundation of human health and organisms interacting in ecosystems provide ecosystem services that are essential to ensure the quality of both natural systems and human welfare. However, these delicate balances are jeopardized by various human-induced pressures, including changes in land use and land cover, pollution, resource overexploitation and climate change.

International programs, like the EU Green Deal, have outlined strategies for sustainable development, emphasizing the priority of enhancing biodiversity and human well-being. However, significant scientific knowledge gaps persist, especially concerning the impact of human activities on biodiversity and ecological interactions. These gaps hinder the effectiveness of ecological restoration efforts in urban and agricultural environments.

In this context, animal-mediated pollination stands out as a compelling investigation target due to its pivotal role in agriculture, food security, and biodiversity conservation. Comprehensive understanding of the dynamics of insect-

plant relationships, particularly in anthropized environments, is fundamental for strengthening pollination processes and generating resilient ecosystems.

This thesis adopts a comprehensive multi-level integrative approach to explore neglected facets of the intricate relationship between environments, insect pollinators, and food security. Specifically, we investigated i) the impact of land use and urban heat island effect on the chemistry of flower rewards (i.e., pollen and nectar); ii) the repercussion of landscape fragmentation and local flower richness on the quality and composition of a generalist pollinator diet; iii) the role of insect mediated pollination on the commercial quality and the nutraceutical value of two phylogenetically distant crops.

The first chapter introduces an innovative tool for pollen sampling (Case study I) and provides a comparative analysis of multiple pollen and nectar sampling techniques (Case study II). The new tool, based on the use of an adapted portable vacuum, is characterized by higher recovery efficiency and precision in isolating pollen grains from other floral tissues, compared to traditional methods. The comparative investigation conducted emphasized the need for standardization of the sampling techniques to produce reliable and shareable data, as significant biases in the chemical characterization were introduced by certain methods, mainly due to ectopic contamination of the pollen matrices.

These methodological advances made the investigation of the impacts of land use and Urban Heat Island on pollen and nectar chemistry easier (Case study III). These

floral resources constitute primary components of pollinators' diet, thus significantly influencing their health status and indirectly contributing to the preservation of efficient pollination service. Flower resources sampled from 7 different meadows species at 16 sites, distributed along gradients of urbanization and agricultural intensification in the metropolitan area of Milan, were analyzed. The study revealed significant effects of land use and temperature on pollen and nectar nutrient profiles. Specifically, agricultural intensification was associated with decreased sugars and increased antioxidant content of flower rewards, while urbanization was positively associated with the total flavonoid content in pollen. The impact of land use was also confirmed through untargeted metabolomic analyses that revealed significant variation in the phytochemical profiles of the pollen of some species along the land use gradients considered.

The second chapter shifts the focus from plants to insects, investigating the nutritional profile and composition of the diet of a generalist pollinator species (*Bombus terrestris*) in fragmented habitats (Case study IV). Commercial colonies of *B. terrestris* were positioned at 14 sites in the city of Milan characterized by different degrees of habitat fragmentation. The nutritional composition of the pollen pellet transported by foragers was analyzed by means of analytical chemistry techniques, providing detailed insights into their macronutrient and phytochemical content. Simultaneously, a description of the taxonomic composition of the pollen was achieved through a High Throughput Sequencing DNA metabarcoding

approach, allowing for a precise identification of plant species contributing to the diet. We found mostly negative linear or non-linear relationships between nutritional quality of pollen loads and habitat fragmentation. The study also revealed tight associations between plant composition and nutrition. Additionally, longer foraging times were observed in areas characterized by a lower amount of green patches. These findings are clear indicators of how habitat fragmentation can pose constraints to bumblebee foraging and compromise resource accessibility, thus impacting on multiple aspects of their nutritional ecology.

Overall, the results presented in the first two chapters emphasize the need for targeted conservation efforts in anthropized environments. Indeed, our research encourages the adoption of local management strategies and landscape planning policies. These initiatives should be aimed at ensuring access for pollinators to both an adequate quantity and quality of resources, thereby enhancing ecosystems functioning and resilience.

In the third chapter, we investigated the direct effects of pollination on plants, specifically examining the influence of insect–mediated pollination on fruit quality and chemical composition (Case study V). Experiments were conducted on two species (i.e., *Fragaria vesca* L. and *Vigna unguiculata* L. Walp.) exposed to three different pollination treatments. Specifically, i) flowers were forced to self-pollinate by covering them with plastic bags, ii) flowers were manually pollinated with the pollen from a different individual, or iii) flowers were left free to be visited by

insects. Higher commercial quality of insect-pollinated fruit was observed and, for the very first time, it was demonstrated that pollination mediated by insects influence the phytochemical profile of fruits and seeds. Wild strawberries originating from flowers pollinated by insects showed higher concentration of many secondary metabolites endowed with nutraceutical properties (e.g., anthocyanins, flavonoids, ellagic acid derivatives,) compared to self- or hand-pollinated ones, while in cowpea most of the secondary compounds occurred at higher concentrations in self pollinated seeds. Notably, in both species, many anti-nutrients such as tannins and saponins were more expressed in self pollination treatments, suggesting possible stress phenomena occurring at the embryo development level. These results have both ecological implications, related for example to seed dispersal and plant defences, and implications for food security and human well-being at the dietary level.

Overall, this thesis advances the current understanding of the intricate relationships between land use intensification, pollinator nutritional ecology, and human food security. The obtained results offer valuable guidelines for the implementation of pollinator monitoring strategies and serve as a clarion call for holistic conservation endeavors, fostering ecosystems resilience and supporting food security and human well-being.

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Part I

Introduction

Challenges for Biodiversity in the One Health Era

In recent years, two fundamental principles regarding biodiversity have emerged and gained consensus within the global scientific community and societal stakeholders. Firstly, biodiversity is recognized as the foundation for human health, and secondly, that organisms interacting in ecosystems are responsible for providing ecosystem services (Millenium Ecosystem assessment, M.E.A, 2005). These services are indispensable both for Earth's life support systems and for the well-being of human societies (Costanza et al., 2020). Although these key concepts are essential to achieving global sustainable development (Wood et al., 2018), biodiversity conservation and services are facing increasing challenges due to many pressures, mainly arising from anthropic activities (Bellard et al., 2022; Maxwell et al., 2016). Among these, land use change (Zimmermann et al., 2010), habitat alteration (Hanski, 2011), overexploitation of natural resources (Hilborn & Sinclair 2021), pollution (Groh et al., 2022), climate change (Habibullah et al., 2022), and the outbreak of invasive species (Duenas et al., 2021; Padayachee et al. 2017) stand first. The primary consequence of this scenario is that the provision of ecosystem services is considered at risk (Boesing et al., 2020).

Concurrently, the increasing awareness of the intimate link between the health of ecosystems and human well-being has also sparked global attention towards the

conservation and restoration of ecosystems. Global initiatives involving governments, academia, and civil society, such as the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES), aim to promote awareness of biodiversity, ecosystems, and their contributions to human societies. This objective finds tangible expression in the Sustainable Development Goals (SDGs), outlined by the United Nations (United Nation-UN, 2015), which stand as a testament to the urgency of aligning human well-being with the preservation of our environmental resources and that recognize the whole biosphere as the foundation of the 17 SDGs. These goals aim to achieve synergy between human well-being and the maintenance of environmental resources by 2030. In this context, the One Health vision recently emerged as a multidisciplinary, integrative, and systemic framework within which the health of people, animals, and the environment (including their reciprocal relationships) must be explored together to derive holistic and effective guidelines or assumptions. This cross-sectoral approach aligns with the general policy strategy outlined in the European Green Deal (EGD), presented by the European Commission in 2019 (European Commission, 2020). The EGD is a crucial part of the EU's plan to achieve the 2030 Agenda for Sustainable Development and its main aim is to “transform the EU into a fair and prosperous society with a modern and competitive economy”. The multidisciplinary approach

at the base of the EGD emerges clearly from the eight key areas that together contribute to the ultimate climate-related goal (Fig. 1).

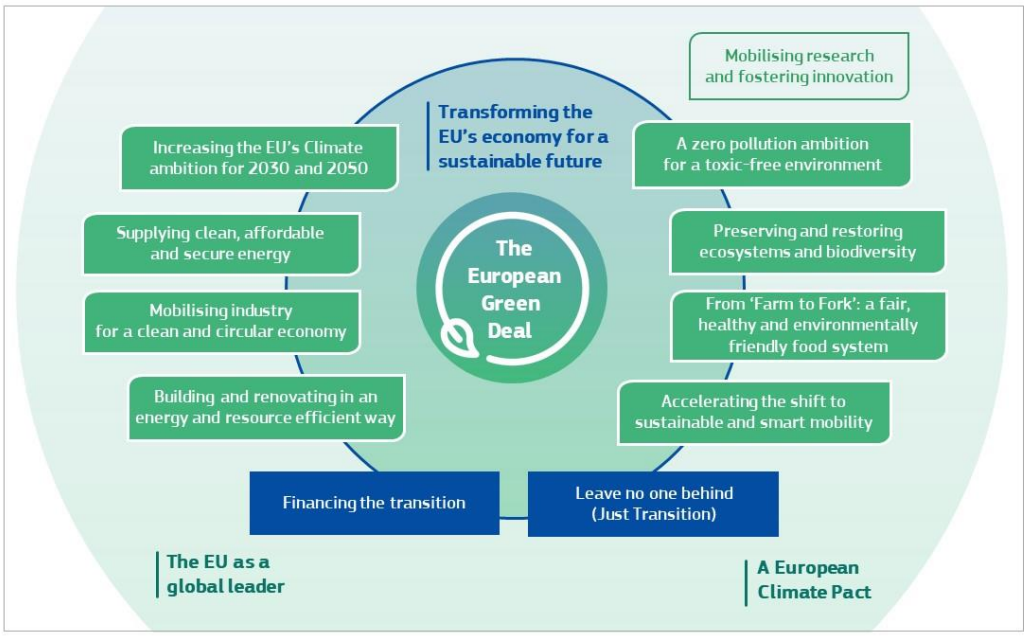


Figure 1. The European Green Deal and the various elements that represent its key areas. Source: European Commission 2019. Communication from the commission. The European Green Deal.

To reach these ambitious goals, the implementation of a legislative framework is urgent. Recent strides in this direction have been made with the landmark "Nature Restoration Law" which proposal was adopted in 2022 by the European Commission (European Commission, 2022). This proposal represents a key step in policymaking toward the restoration of ecosystems, habitats, and species, standing as the first continent-wide, comprehensive law of this kind. The Nature Restoration

Law is integral to the EGD, and its main goals are to enable long-term and sustained recovery of biodiverse and resilient nature, meet international commitments, and contribute to achieving the EU climate mitigation and adaptation objectives. These are translated into specific, legally binding targets for Member States, which will be asked to formulate national restoration plans, outlining the projects and initiatives they intend to pursue to achieve the EU target. Member States will be required to put in place effective restoration measures that cover at least 20 % of the EU's land and sea areas by 2030 with particular emphasis on urban areas and agricultural ecosystems. Specifically, measures will have to be taken to increase high-diversity features on agricultural lands (such as hedgerows and flower strips) and to increase green spaces and the amount of tree cover in cities. This focus on human-altered landscapes is not only due to their significant role in driving biodiversity loss, but also because they offer substantial opportunities for implementing effective management strategies. In this political framework it must be underlined that the concept of restoration must not only be interpreted in a radical way, (i.e., reforestation actions and urban greening plans), but also as strategies to improve the functionality of ecosystems by promoting resilience and evolution.

In this context, the demand for harmonized and innovative monitoring strategies to track project progress and validate goals attainment is essential. These will involve the development of tools for the collection and management of biodiversity

data that underpins scientific knowledge production. This urgency has been underscore, for example, by the Biodiversa+ call launched in 2022 that deals with "Improved translational monitoring of biodiversity and ecosystems change for science and society" (https://www.biodiversa.eu/2022/10/07/2022-2023-joint-call/). In this framework, the world of animal-mediated pollination emerges as one of the most promising bioindicators. Pollinators provide a range of ecosystem services (Gill et al., 2016; Matias et al., 2017) and with their far-reaching impacts on agriculture, food security, and biodiversity conservation, they stand as a compelling investigation target. Among the vast plethora of pollinator animals, bees contribute directly to various SDGs, including food security (SDG2) and biodiversity conservation (SDG15). Beyond these direct contributions, bees have the potential to impact a broader spectrum of SDGs target, as highlighted by Patel and colleagues (2021), who identified connections between bees and 30 specific SDGs targets (Fig. 2). The importance of pollinators is also highlighted in the EGD and Nature Restoration Law which set goals for the reversal of pollinator declines by 2030.

Overall, the investigation of pollination ecology provides a unique opportunity to develop new biodiversity monitoring strategies, validate the efficacy of territorial management policy, and ultimately draw a comprehensive picture of the profound connections and interdependency of environmental, animal, and human health.

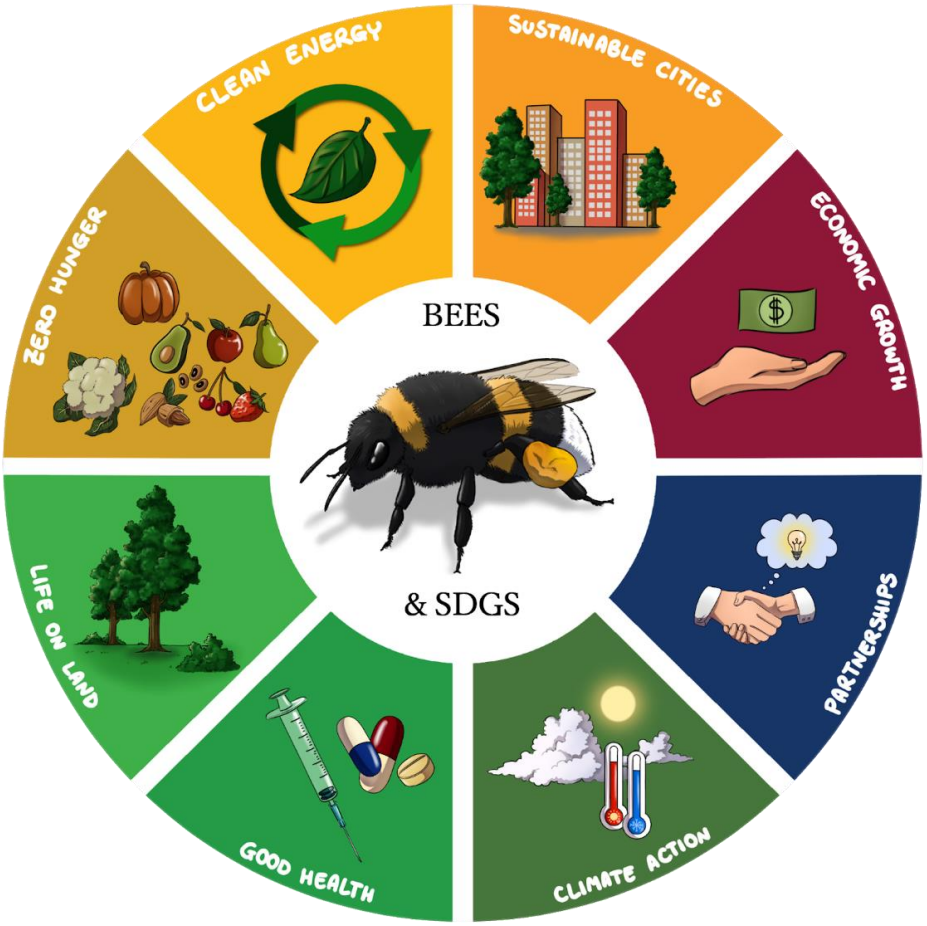


Figure 2. The figure represents the overarching themes in which pollinators can contribute to sustainable development goals.

Pollination Ecosystem service

Among the multiple ecosystem services to which pollinators directly or indirectly contribute, pollination represents the main one. Animal-mediated pollination is essential for the reproduction of both wild and cultivated flowering plants (Ollerton et al., 2011; Potts et al., 2010), and maintaining plant-pollinator interactions is the basis for the function and sustainability of healthy ecosystems. Recent estimates by Tong et al. (2023) indicated that 90 % of flowering plants rely on animal-pollination (Fig. 3), surpassing even the previous estimate made by Ollerton et al. (2011) according to whom it ranged between 85% and 87.5%.

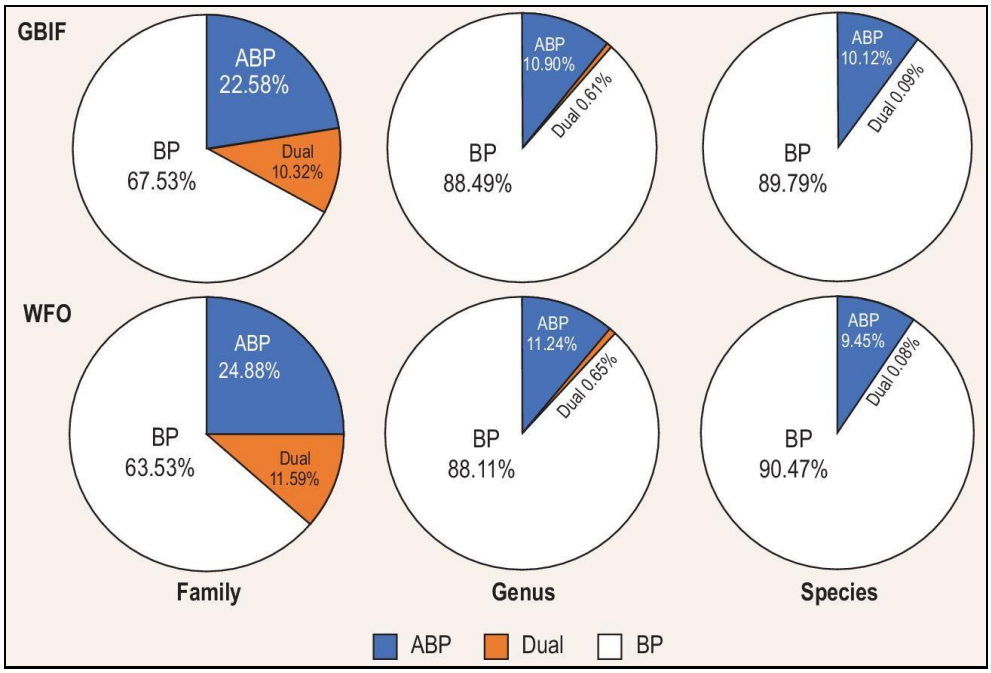


Figure 3. Percentages of biotic (BP), abiotic (ABP), and ambophilous (Dual) pollination based on the results of the literature search by Tong et al. 2023 and the use of the most complete angiosperm name databases GBIF and WFO (accessed in February 2023).

These data pinpoint the crucial importance of pollinators conservation, especially insects, representing the most widespread group in the pollinator guild (Ollerton, 2017). Bees, despite not being the most species-rich group among insect pollinators, dominate in almost every region except for the Arctic ones, where flies prevail (Ollerton, 2017). Bees are also among the most efficient pollinators due to their ability to transport a large number of pollen grains and their heavy reliance on floral resources compared to other pollinators (Ollerton, 2017; Jhonson & Anderson, 2010). Other insect orders such as Lepidoptera, Diptera, Coleoptera, and non-bee Hymenoptera contribute significantly to the provision of the ecosystem service of pollination (Rader et al., 2020). Among dipterans, the Syrphidae family for example, is one of the major groups of non-bee flower visitors and visits a comparable proportion of flowers to those visited by Hymenoptera (Klecka et al., 2018).

The undeniable connection between pollination and human well-being is underscored by its incontrovertible economic value, primarily derived from its role in agricultural production and food security (IPBES 2016). Pollinator-dependent crops contribute to about 35% of overall crop production by volume (IPBES 2016), and crop pollination service has a value estimated to range from US$195 billion to ~US$387 billion annually (Porto et al., 2020). Acknowledging the significance of pollination for modern societies is a pivotal issue, especially considering recent

assessments revealing declines in wild pollinators in both Europe and North America (Potts et al., 2010). Despite the urgency posed by these declines, data shortfalls and the absence of comprehensive monitoring programs persist in many regions worldwide (Timberlake and Morgan, 2018). This knowledge gap becomes even more apparent when considering the limited evaluation of the global conservation status of bee species, with a significant proportion categorized as "data deficient" (IUCN, 2019). This alarming situation has been effectively highlighted by Nieto et al. (2014), who pointed out the high likelihood that a substantial number of unstudied species might also be under threat. This concern is echoed by the research conducted by Goulson and colleagues (2015), who argued that the declines observed in Europe and North America are probably indicative of similar trends in other unexamined regions across the globe. This situation underscores the urgency of comprehensive research and conservation efforts to address the global decline of pollinator populations.

In the last decades, there has been a surge in research aimed at identifying the drivers of this trend and the subsequent impacts on pollination service (Dicks et al., 2021; Goulson et al., 2015; Decoury et al. 2019). However, numerous underlying mechanisms remain poorly understood. Clarifying these intricate mechanisms is essential for implementing effective conservation strategies and safeguarding the essential role of pollinators in our ecosystems.

As the human-induced land use and land cover change (LULC) represents the primary cause of pollinators decline, there has been an increase in public, political, and scientific interest in the sustainable management and development of agricultural and urban areas, which are the main drivers of landscape anthropization (Lau et al., 2023; Seto et al. 2012; Van Klink et al., 2020). Agricultural production covers about 35% of the earth's terrestrial surface (Lau et al., 2023), while cities, despite covering an extremely small percentage of the global land, contribute to 90% of the economic output and 70% of global greenhouse emissions (Solecky et al., 2013). These data underscore the pivotal role played by these anthropogenic landscapes in driving global change phenomena. Furthermore, the interest in these areas is also driven by recognizing that despite the biotic and abiotic pressures they pose to biodiversity conservation, they may potentially represent biodiverse ecosystems (Lau et al., 2023; Ayers & Rehan 2021).

Insect pollinators in human-altered environments

Pollinators habitats encompass diverse environments, including natural, semi-natural landscapes (e.g., hedgerows), and anthropic areas (e.g., agricultural, and urban areas). The preservation of the integrity and health of these habitats is of utmost importance and plays a pivotal role in safeguarding the benefits that humans receive from pollinators, ultimately impacting our overall well-being. Human-driven alterations in land use and land cover encompass several factors that pose a significant threat to pollinator preservation (Wenzel et al., 2020; McDonald et al., 2020; Lau et al., 2023). Habitat loss, pesticide exposure, the presence of invasive species, and climate change, which are all closely linked to landscape anthropization are key drivers of the decline we are witnessing (Potts et al., 2016). Agricultural intensification and the expansion of urban areas are distinct phenomena but share some common characteristics. Both are guided by the rapid growth of the global human population that creates demands for food and housing resources. The creation of large extensions of monoculture crops and the increase in impervious surfaces that characterize agricultural and urban landscapes respectively have as prominent outcomes the loss and fragmentation of habitats (Kovács-Hostyánszki et al., 2017; Senapathi et al., 2017). Beyond this primary consequence, these phenomena impact pollinators at multiple levels, from the reduction of flower diversity and abundance due to the high use of agrochemicals

and the intensively performed tillage, grazing, or mowing (Aviron et al., 2023; Lerman et al., 2021; Bretzel et al., 2016), to the loss of nesting habitat due to the decrease in bare-ground patches (Wilson & Jamieson, 2019). These pressures can shape the plant and pollinator community composition (Xiao et al., 2016) and their interactions (Matthews et al., 2014). Cities are also characterized by changes in the climate due to urban warming or the urban heat island (UHI) effect (Deilami et al., 2018) and by an increased occurrence of environmental contaminants (e.g., soil and air pollution, noise, artificial light). All these elements interact with each other, covary with the progress of urbanization, and can influence ecosystems functioning at either the landscape or local scale (Fig.4; Ayers and Rehan, 2021).

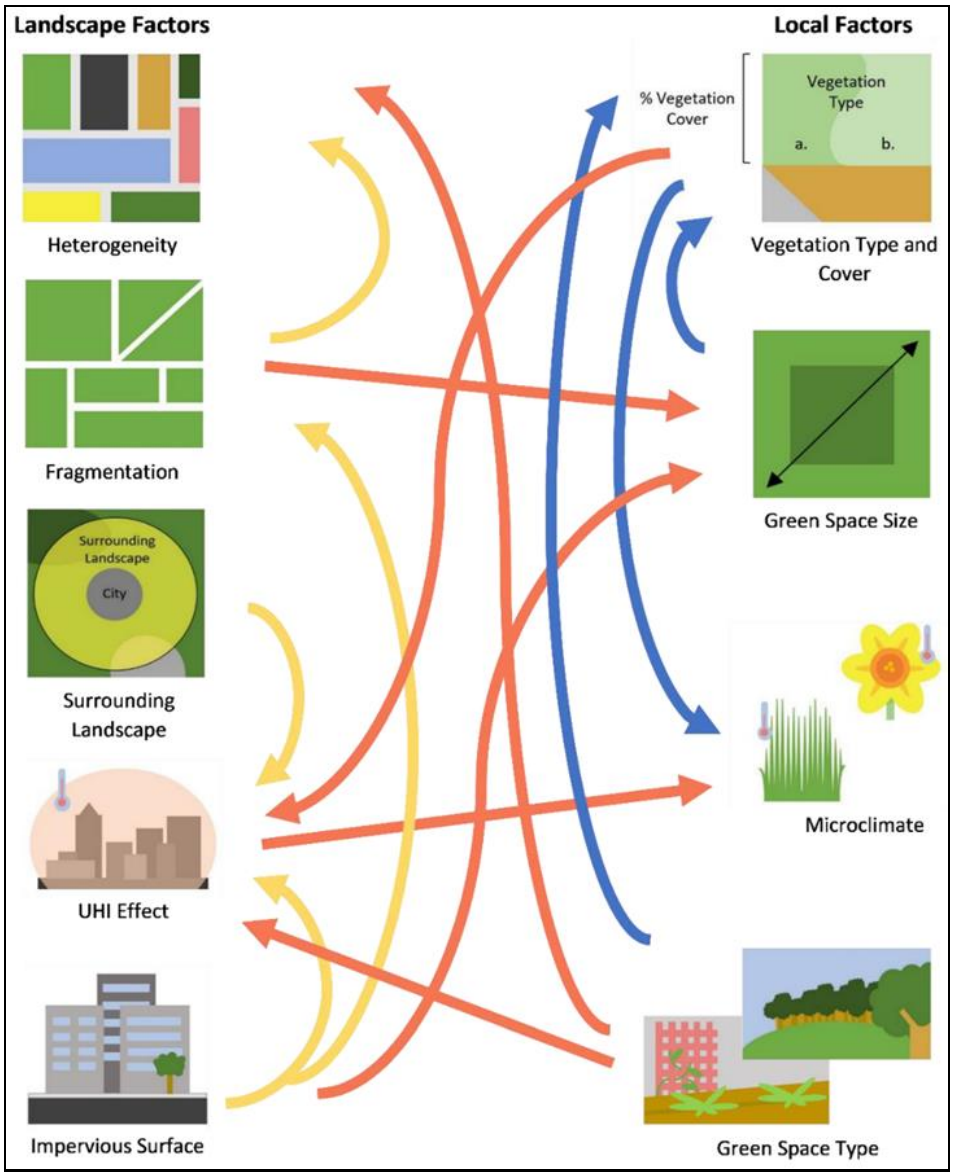


Figure 4. Diagrams illustrating interactions between landscape and local features associated with urbanization. The arrows denote features influenced by other features originating the arrow. The diagram distinguishes between local-local interactions (depicted in blue), landscape-local interactions (depicted in red), and landscape-landscape interactions (depicted in yellow) (Ayers & Rehan, 2021).

Due to these set of conditions the expansion of cities, in conjunction with intensified agriculture, has often been indicated as the major determinant of the declines in biodiversity, species interactions, and ecosystem services (Grimm et al., 2008, Beninde et al., 2015; Grimm et al., 2008; McKinney, 2008; Piano et al., 2020). Land use influences many ecological processes that rely on species interactions, including for example seed dispersal and frugivory (Palacio & Ordano, 2023), and herbivory (Miles et al., 2019, Santangelo et al., 2022), and particular concern has been raised about its impact on pollinators' conservation (Wenzel et al., 2020).

Although these characteristics can lead to the conclusion that agricultural areas and cities are only detrimental to pollinator conservation, their real impact is not so obvious (Lau et al., 2023; Spotswood et al., 2021, Theodorou et al., 2020). The configuration of agroecosystems, typically characterized by monocultures dominating a landscape and a mosaic of smaller natural and seminatural areas create a system of ecological corridors playing an important role for pollinators (Zhang et al., 2023). Crop plants themselves may offer a significant amount of floral resources although limited in time and space (Lau et al., 2023), and agroecosystems can also provide additional resources, such as nesting habitat and trophic rewards deriving from the wildflowers community (Kim et al., 2006; Arathi et al., 2018). Recent studies have also revealed how urbanization can also have a neutral or even positive impact on both pollinators' abundance and richness (Millard et al., 2021;

Theodorou et al., 2020; McDonald et al., 2020; Wenzel et al., 2020). This observation can be partly explained by the fact that the richness of pollinators may initially increase in response to intermediate levels of disturbance (e.g., Vulliamy et al., 2006), but it rapidly declines as the level of disturbance becomes intense (Chacoff & Aizen 2006). Applying this rule directly to assess the impact of urbanization on pollinator communities proves challenging due to the varied effects experienced by different taxonomic and functional groups of pollinators (Wenzel et al., 2020; Ayers & Rehan, 2021). For example, ground-nesting pollinator species that require patchy vegetation characteristic of early successional stages are likely to benefit from a moderate level of disturbance (Kremen et al., 2007; Vulliamy et al. 2006) while in a highly urbanized context, cavity-nesting species will be favored due to the lack of bare-soil nesting sites (Ayers & Rehan, 2021). Moreover, the urban matrix seems to benefit small, social, polylectic, and non-native species (Ayers & Rhean 2021; Bucholz & Egerer, 2020; Fitch et al., 2019), so that it can be seen to behave as a filter on biotic communities and on pollinators species (Ayers and Rehan, 2021; Aronson et al., 2016). Recent evidence also indicated that Hymenoptera are more resilient to urbanization compared to Diptera or Lepidoptera (Baldock et al., 2015; Fenolgio et al., 2020; Theodorou et al., 2020). The complex and diverse effects of urbanization on pollinator communities have triggered significant interest in the field of pollination ecology in the context of

anthropized environments. This topic extends beyond recognizing the negative impacts of anthropic pressures and involves the evaluation of the potential of cities to serve as refugia for pollinator species (Baldock, 2020; Hall et al., 2017). Although we have a growing understanding of how urbanization impacts pollinator communities, it is unclear whether the observed changes can translate into a shift in pollination service provision (Liang et al., 2023; Theodorou et al., 2022). The variation in the pollinator communities' structure and functional diversity could indeed impact plant fitness, by reducing the efficiency or frequency of pollen transfer, ultimately leading to pollen limitation phenomena (Irwin et al., 2018). Despite such a detrimental impact of urbanization has consistently been detected, the expansion of urban areas may not always affect pollinator abundance and pollination service. Its filter effect can indeed suppress threatened or rarely observed species but favor generalist and managed bees. Indeed, a recent meta-analysis by Liang et al. (2023) has revealed that while urbanization has a negative impact on pollinator abundance and richness, this effect does not clearly translate into a decreased pollination service, due to the presence of abundant generalist and managed pollinators in urban areas.

To comprehensively unravel the intricate relationship among pollinators, land use changes, and the ecosystem service of pollination, all facets of pollination ecology in anthropized environments must be thoroughly addressed. A pivotal aspect to

investigate is the interaction existing between the two primary actors in this context: the pollinators and the plant communities. The transformations of natural environments driven by urbanization and agricultural intensification can indeed accelerate evolutionary selections and reshape fundamental ecological interactions (Palacio & Ordano, 2023), including the mutualistic relationship between plants and pollinators. The decrease in pollinator diversity often observed in these landscapes represents a potent driver of evolutionary changes in the plant communities, as well pinpointed by various studies. In the meta-analysis of Liang et al., 2023, for example, a selection towards radial flower morphology in urban areas was highlighted and this observation was explained by the fact that this floral shape can benefit from the presence of generalist pollinators that are usually favored in these habitats. The increased proportion of generalist pollinators and the high prevalence of managed species in cities can also be the base for the prevalence of exotic or invasive plant species due to the documented preference of these pollinators' functional groups for exotic flowering species (Goulson et al., 2003).

A large amount of research has been dedicated to investigating patterns of rewiring of the plant-pollinator networks in anthropic environments (Biella et al., 2022; Prendergast & Ollerton, 2021; Theodoru et al., 2017). Despite these efforts, critical aspects that could offer a unique perspective on this topic, such as those linked to pollinators nutrition have been overlooked until recently.

Impact of land use on pollinators nutrition

Pollinators exhibit diverse dietary patterns. Nutritional requirements vary widely not only at the inter-specific level but also at the intra-specific one, with individuals of different life stages and sexes showing different requirements (Altaye et al., 2010; Human et al., 2007). For instance, fly larvae (Diptera) are predominantly detritivores or predators (Davis et al., 2023) and larvae of Lepidoptera species are primarily herbivorous, often displaying high levels of specialization (Altermatt et al., 2011). Despite these diversities, all pollinators share a fundamental partial or complete reliance on pollen and nectar as food sources (Nicolson et al., 2018). Bees stand out as a remarkable example as almost all the species exclusively rely on flower resources (Brodschneider & Crailsheim, 2010).

Pollen and nectar contain proteins, carbohydrates, and lipids that belong to the class of macronutrients and represent the major portion of the nutritional intake in all organisms (Biesalski, 2017). They also contain vitamins, dietary minerals, and secondary metabolites. These micronutrients play crucial roles in diverse biological processes from osmoregulation to immune system functions (Glavinic et al., 2017). Pollen and nectar display relevant differences in their chemical composition. Nectar is essentially a solution of sugars, but also contains amino acids and phytochemicals (Barberis et al., 2021). Compared to nectar, pollen chemistry is more

heterogeneous. It contains proteins, lipids, amino acids, and a higher diversity and concentration of secondary metabolites compared to nectar (Palmer-Young et al., 2019).

The balanced intake of macronutrients and micronutrients is crucial for pollinator fitness. Deviations from the optimal nutritional intake, the so-called “nutrient target” can lead to severe consequences such as deficient growth (Vaudo et al., 2018), compromised immune functions (Di Pasquale et al., 2013), and reduced reproductive success (Schweiger et al., 2022). Not only the absolute intake but also the relative proportion of these nutrients in the diet is vital for determining the health status of pollinators (Vaudo et al., 2016). The importance of a balanced assumption of macro- and micronutrients has been emphasized by evidence from several bee species showing clear food choices of floral resources to meet their nutritional demands (Kriesell et al., 2017; Liu et al., 2006; Ruedenauer et al., 2016; Vaudo et al., 2016). Pollen macronutrients play a key role in this food choice, as indicated by experiments showing that *Bombus terrestris* and *B. impatiens* workers can regulate their protein and lipid intake preferring pollen with higher protein concentration while avoiding provisions too rich in lipids (Vaudo et al., 2016). Beyond single nutrient concentrations, the ratios of macronutrients are also pivotal to describe bees foraging behavior, such as the protein:lipid ratio (P:L) as highlighted by a recent study by Kraus et al. (2019) that demonstrated how entire

bumblebee colonies selectively forage to reach an optimal ratio of proteins, lipids, and carbohydrates. A similar pattern of active food choice has also been found at the community level in the experiment by Finkelstein et al. (2022) that documented an increase in pollinator visitation rates and higher diversity of visiting pollinator species when the nectar of wild species was enriched with Na. Phytochemicals that can act as protective agents, such as antioxidants, antibiotics, and detoxifiers (Barascou et al., 2021; Wong et al., 2018) also have a role as attractive agents or deterrents. The most evident way through which secondary metabolites exert this function is through visual signals. For example, blue colors exhibited by certain flowers are produced by anthocyanins such as delphinidins (Miller, Owens, and Rørslett, 2011) and serve as strong attractants for many pollinator species (Reverté et al., 2016). Caffeine occurring in the nectar of many plant species has the potential to enhance memory retention of pollinators thus increasing floral focus (Koch and Stevenson, 2017), but it exerts toxicity upon specific dosages (10^{-4} M). It is important to underline that most of the studies performed on the effect of phytochemicals on bee health underlined the fact that the beneficial effects of these compounds are gained within specific doses, such as the case of p-coumaric acid that act as a pesticide detoxifier (Mao et al., 2023) or polyamines that can mitigate oxidative stress related phenomena (Dordievski et al., 2023). Disproportionate concentrations of these compounds are usually related to toxic

effects (Stevenson et al., 2017). An interesting situation is also represented by the plants belonging to the family of Asteraceae whose pollen is seldom foraged by polylectic species (e.g., *B. terrestris*), despite them representing one of the most widespread and abundant groups of plants. This phenomenon is partly due to the presence of toxic phytochemicals in the pollen that act as a chemical defence from herbivory (Vanderplanck et al., 2020). Many studies are trying to disentangle the reason for which floral rewards may contain deterrent compounds and many hypotheses are still debated nowadays. Some researchers argue that this phenomenon may be due to a sort of heritage coming from vegetative tissues, such as leaves, where the production of defence secondary compounds is essential to protect against herbivores (Bennett & Wallsgrove, 1994). According to this theory, the occurrence of secondary compounds in floral rewards may be a trait under evolutionary pressure. Other hypotheses suggest that the presence of secondary compounds in floral rewards is important to avoid the spread of opportunistic microorganisms, that may waste the rewards provided by plants to pollinators (Stevenson, 2020). Notably, this intricate relationship between pollinators and plants, shaped by the chemical composition of floral resources, adds further layers of complexity to their interactions. In the context of landscape anthropization and its multifaceted impact on pollinator ecology, these nutritional dynamics assume paramount importance.

Both biotic and abiotic conditions within the human-altered landscape can influence various aspects of pollinators' nutritional ecology (Hülsmann et al., 2015; Winfree et al., 2011). The alterations in landscape composition and configuration, associated with the increase in impervious cover and intensive agriculture, lead to lower connectivity between green patches (Wenzel et al., 2020) and a reduction in their size, diminishing their quality intended as the potential to sustain local populations of pollinators (Fahrig, 2003). These conditions shape both the plant and pollinator communities' structure and their interactions (Grass et al., 2018), significantly impacting the pollinator's nutritional ecology (Gervais et al., 2020; Leach & Drummond, 2018; Theodorou et al., 2020). Land use intensification is often associated with diminished floral diversity and abundance (Gossner et al., 2016; Potts et al., 2010), altered plant community composition (Requier & Leonhardt, 2020; Weiner et al., 2011), and variations in the spatial distribution of floral resources (Matteson et al., 2013). Although large crop fields and monocultures can represent a relevant trophic resource, their availability is limited only to the flowering period of the crop (Straub et al., 2023). The uneven distribution of plant resources for pollinators is also evident in the linear decrease of pollen diversity collected by pollinators across a gradient of green areas fragmentation in urbanized areas (Biella et al., 2022). Moreover, fragmentation directly impacts pollinators' foraging behavior (Gervais et al., 2020). According to the optimal foraging theory,

pollinators tend to forage closer to their nesting sites in a landscape characterized by lower habitat fragmentation and even resource distribution to reduce their energetic expenditure (Goulson, 1999). This was corroborated by a study demonstrating that landscapes with higher green coverage were associated with shorter foraging distances and trip duration in several bumblebee species (Redhead et al., 2016). While numerous studies investigated variations in food resources directly collected by a few model species in response to landscape management and anthropization gradient (Pioltelli et al., 2023; Vaudo et al., 2018; Donkersley et al., 2017), low attention has been directed to investigating the nutritional landscape represented by plant communities and the potential variation in the chemical composition of floral rewards due to environmental pressures (Biella et al., 2022; Venjakob et al., 2020) that can ultimately impact the quality of pollinators' diet (Bucholz & Egerer, 2020).

The chemistry of floral rewards depends mainly on phylogenetic signals but is also influenced by environmental variables, especially in the quantitative variation of phytochemicals (Zu et al., 2021; Palmer Young et al., 2019). Indeed, the metabolism of plant secondary compounds is known to be highly responsive to environmental factors among which light, temperature, and drought, as well as biotic agents, such as the damage produced by herbivores and parasites (Khare et al., 2020). The quality and quantity of plant resources can be affected for example by fertilizers,

widely used in agriculture (Fox, 2013). The limitations imposed by landscape composition and configuration on pollinator foraging behavior and flower resource availability, coupled with potential variations in their chemical composition, pose a significant threat to urban pollinators population conservation and the ecosystem service they provide.

This concern is particularly alarming considering the escalating importance of urban and peri-urban agriculture, especially in developing countries, due to their substantial social and economic benefits (Wenzel et al., 2020). Recent assessments have underscored the significance of this practice, indicating that 15-20% of global food production originates from these types of agriculture (Orsini et al., 2013; Abdulkadir et al., 2012) and these estimates are constantly increasing, coherently with the intensification and expansion of urban areas.

The role of animal pollination for food security

Most calories in the human diet are derived from a limited number of pollinator-independent staple crops, such as rice, wheat, potato, and corn (Ghazoul, 2005; Richards, 2001). These crops primarily rely on wind pollination, self-pollination, or vegetative propagation for reproduction. Despite their high energetic value, these crops are deficient in most micronutrients (Eilers et al., 2011). In contrast, pollinator-dependent crops, accounting for only 2.5 % of the global calories production (Garibaldi et al., 2022), play a disproportionate role in dietary nutrient supply (Smith et al., 2015; Kramer et al., 2014). Indeed, animal pollination is responsible for up to 20 % of vitamin C, 41% of vitamin A, and 7% of folate supplies globally (Eilers et al., 2011). Additionally, oils from plants promoted by animal pollination constitute approximately 74% of globally produced lipids (Eilers et al., 2011). Animal-pollinated plants also contribute significantly to mineral provision in the human diet, with 58% of calcium and 62% of fluoride derived from plants whose yields are at least partially influenced by animal pollination (Eilers et al., 2011). The important role of pollination for food crops is largely acknowledged (Dainese et al., 2019; Gallai et al., 2009) and growing concerns are expressed regarding the potential impact of their decline as the demands for pollination service is already outstripping the supply. The importance of animal pollination is highlighted further

considering that micronutrient deficiencies, often referred to as “Hidden hunger”, affect over 25% of the global population (International Food Policy Research Institute, (IFPRI), 2014; Willet et al., 2019; Nicole, 2015, Lim et al., 2012). The condition, affecting individuals of all ages and genders, can occur in individuals who have no energy deficits and even coexists with obesity cases (Gödecke et al., 2018). It can lead to increased incidence of chronic (NCDs) and infectious diseases, contributing to a global burden of elevated morbidity and mortality (Willet et al., 2019). Developing regions are particularly endangered due to their higher reliance on animal pollination (Millard et al., 2023), limited data on pollinators status (Goulson et al., 2015), and existing vulnerability to food and nutrient shortages related to demographic and climate change (Muhammad et al., 2017).

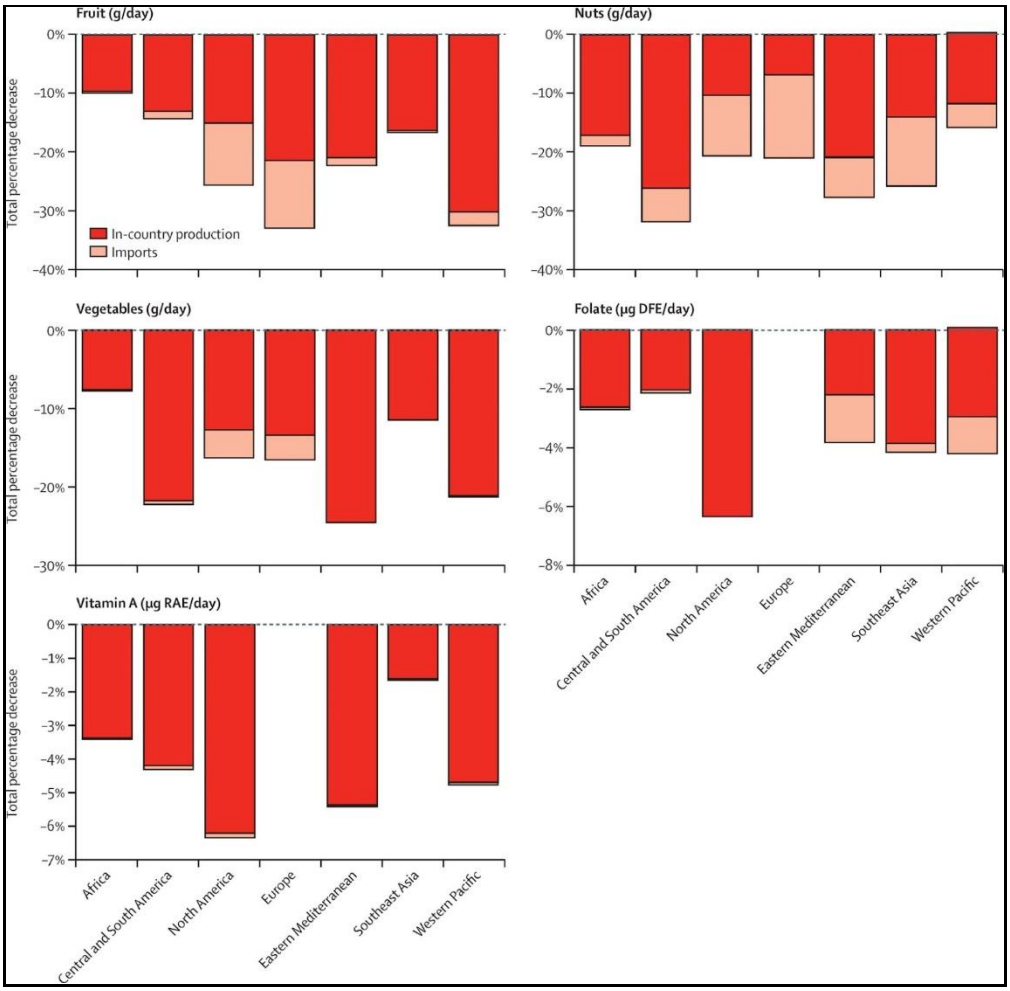


Figure 5. Decreases in food and nutritional intake with full pollinator removal. Source: Smith et al., 2015.

Though the contribution of pollinators to human nutrition through their role in increasing crop yield has already been recognized (Garibaldi et al., 2013; Klatt et al., 2014), whether animal-mediated pollination is able to influence the nutritional quality of agronomic crops remains a relatively understudied area (Wietzke et al., 2018). Actually, although the Green Revolution and the intervention of GMOs in the agriculture scenario made it possible to select for a wide range of crop varieties able

to self-pollinate irrespective of their pollinator vectors, there is growing evidence that insect mediated pollination can affect the nutritional quality of specific foods, as shown for instance for apples (Garratt et al., 2014) and oilseed rape (Bommarco et al., 2012). Recent studies have observed higher sugar and acid concentrations in strawberries (Wietzke et al., 2018; Rosianski et al., 2016) or variations in the ratio of mono- and polyunsaturated fatty acids in almonds (Brittain et al., 2014) produced by flowers visited by insects, or even higher production of anethol (an essential oil) in fennel seeds originating from insect mediated pollination compared to self-pollinated ones (Schurr et al., 2022). These findings suggest that pollination mechanisms possess the capability to initiate or shape a wide array of plant metabolic pathways that ultimately determine the chemical composition of food. In Wietzke et al., 2018, the higher fertilization success due to insect mediated pollination translated into increased levels of auxins that explained the difference in size and shape observed in insect pollinated strawberries compared to self-pollinated ones.

However, to date, no study investigated the influence of pollinator insects on the metabolism of fruit and seed relevant to human nutrition, especially concerning the investigation of secondary metabolites of recognized nutraceutical importance (e.g., phenolic compounds, terpenes, carotenoids, etc.). Overall, a more precise investigation of the relationship between insect-mediated pollination and the

chemical features of agronomic products has the potential to pave the way for novel policy strategies aimed at promoting biodiversity conservation, facilitating the ecological transition of productive systems, and enhancing human well-being. These objectives align with the priorities delineated in the National Recovery and Resilience Plan (PNRR) and the directives of the EU Green Deal. By unravelling the influence of insect mediated pollination on the nutritional value of crops, future research has the potential to enhance the recognition of biodiversity as a fundamental pillar for human health.

Objectives

The two main scopes of this PhD project were:

i) to provide a multi-level assessment of the impact of human-induced land use intensification (and related stressors) on the relationships between plants and pollinators, with specific attention to the flower resources (i.e., pollen and nectar) and the pollinators nutritional ecology. This investigation strategy offers the opportunity to identify suitable mitigation strategies and to implement actions aimed at strengthening the functional biodiversity, in contexts of high anthropogenic pressures.

ii) to delve into the mechanisms of plant-pollinator interactions, with reference to the role of the insect in modulating the evolution of the ovary into fruit. This last element is relevant both for the reproductive and dispersion success of the plant, and to understand the implications of pollination mediated by insects in agricultural contexts. Specifically, evidence was sought to demonstrate the influence of animal pollination on the commercial quality and chemical composition of plants fruit and seed.

To achieve these ambitious objectives, the work was developed through specific case studies, carried out in an operational environment, and using integrated approaches based on field observations, biomolecular analyses and analytical chemistry.

The project also included a study carried out in a controlled environment which made it possible to evaluate the effects on the fruits of the plants in the presence and absence of pollinators. The project was therefore divided into consequential phases that pursued intermediate goals.

• Assessment of the impact of landscape composition and UHI effect on the chemistry of pollen and nectar foraged by pollinator insects. This involved a detailed analysis of land cover and temperature data in anthropized contexts with the aim of determining a measurable cause-effect relationship in terms of macro and micronutrients content variations in nectar and pollen. The study was carried out by considering gradients of increasing landscape anthropization (i.e., agricultural intensification and urbanization).

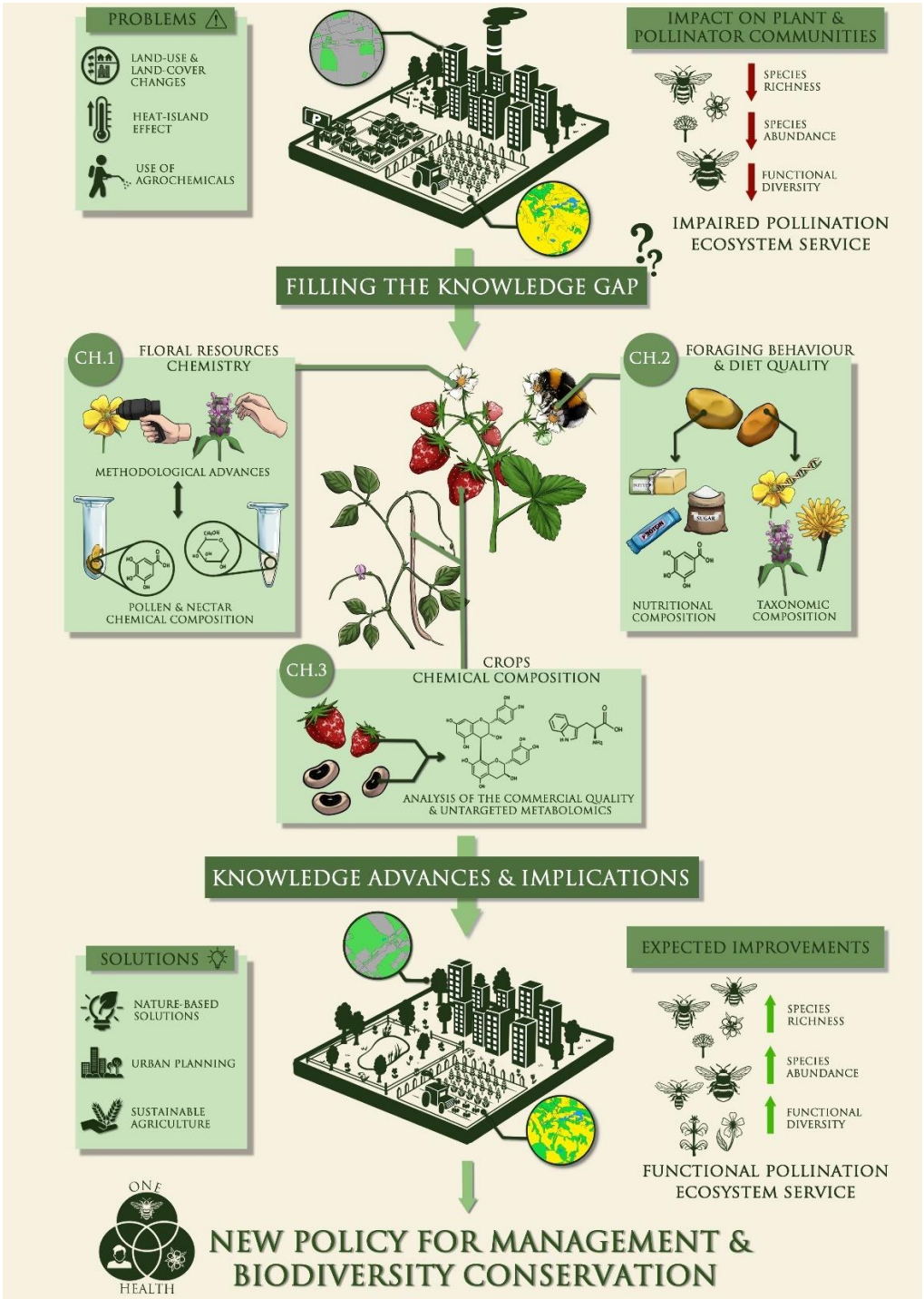
• Investigation of the impact of green habitat fragmentation and local features (e.g., flower richness) on the nutritional profile and composition of the diet of a generalist pollinator species, and on its foraging behaviour. This second goal went into the merits of the functional relationship between plant and pollination from the point of view of resources and the effects on the well-being of the insect. It should also

be underlined that this second objective was pursued in an operational environment by analyzing urban and peri-urban study areas characterized by different degree of green areas fragmentation.

• Assessments of the role of insect-mediated pollination in shaping fruit and seed morphological and nutritional/nutraceutical value. In this context, the aim is to evaluate how insect-mediated pollination can influence the chemical composition of food. This last aim is undoubtedly the most ambitious and innovative as it intends to study a level of 'communication' promoted by the insect towards the plant through the evaluation of the final product of the pollination and fertilization process and specifically the development of seed and fruit.

The results obtained represent valuable information to guide and develop reliable policy guidelines, useful for territory management actors, to achieve conservation and sustainability issues.

Experimental design



Methods

The experimental designs presented in the different chapters of the thesis involved multiple methodological approaches mainly related to the contexts of sampling, wet-lab analytics, and statistical approaches for data interpretation. In this section, a general but comprehensive overview of the various methodologies is presented. Detailed protocols and data analysis pipelines are provided in the Chapters of Part II.

Field sampling

The studies presented in the thesis encompass various sampling approaches, including field sampling of flower rewards (i.e., nectar and pollen) and/or of pollinator insects (Chapters I, II) and sampling of plant portions cultivated under controlled conditions (Chapters I, III).

In Chapter I, a study on the chemistry of pollen and nectar is presented (Case study III). A standard sampling technique was employed for nectar retrieval, involving the use of glass microcapillaries of different volumes. For pollen sampling, an innovative tool was developed and extensively described in Chapter I (Case study I-II). The tool consists of an adapted portable vacuum cleaner equipped with lab tube and filter systems, allowing non-invasive pollen collection and its isolation from other floral parts. This new tool was designed to address the necessity of

standardizing pollen sampling as the wide panel of techniques found in the literature results in great heterogeneity among the different studies, making the outcomes difficult to compare. Furthermore, the need to collect enough pollen for subsequent chemical analyses requires an efficient method characterized by the universality of use among different flower morphologies. The development of the tool and its comparison with other pollen sampling methods is extensively described in Chapter I.

In Chapter II, the experiment involved the positioning of commercial colonies of *Bombus terrestris* at different sampling sites in the metropolitan area of Milan, characterized by different level of landscape fragmentation (Case study IV). Pollen load sampling was carried out by catching foragers returning to the colony with entomological nets. The foragers were then transferred to small plastic bags, and the pollen pellets were removed from the corbiculae using sterilized tweezers.

Cultivation and experimental treatments

The study in Chapter III involved the cultivation of two target plant species in both greenhouses (*Fragaria vesca* L., commonly known as “wild strawberry”) and open field (*Vigna unguiculata* L. Walp., known also as “cowpea”), with the collaboration of the CREA Institute of Sanremo, which hosted the field activities and supported the sampling activities (Case study V). Significant effort was dedicated to

conducting the experimental treatment outlined in the chapter. These treatments included the use of specific plastic bags for self-pollination and the tool developed in Chapter I and brushes for hand pollination. Plants of *F. vesca* were cultivated in open greenhouses while *V.unguiculata* was cultivated in the field. The experiments were conducted along two cultivation season (2021-2021). Irrigation and fertilization regimes were standardized for each plant to avoid biases in the subsequent analysis of the fruits and seeds produced.

Selection of sampling sites

The first step in studies presented in Chapters I and II was the sampling sites selection. Specifically in Chapter II, the selection was made to cover gradients of agricultural intensification and urbanization (i.e., from semi-natural to rural and urban areas), while in Chapter III the gradient aimed at describing as better as possible landscape configuration, with a specific focus on habitat fragmentation. Data on land cover were retrieved from regional land use cartography available for the study area. These data were then processed by using GIS software and the package *"landscapemetrics"* in R (Hesselbarth et al., 2019) to extract information on landscape composition and configuration. Briefly, multiple indexes of landscape fragmentation were computed (i.e., "% Green Cover", "Edge density", "Euclidean nearest neighbour distance", "Contagion").

Environmental features were also investigated for their possible impact on plant resource chemistry in Chapter II. Data on land surface temperature (LST) were recovered through a remote sensing approach. Specifically, data from the MODIS sensor database (https://modis.gsfc.nasa.gov/) were downloaded. Data on the daily mean temperature registered during the sampling period were obtained from the database and were then processed to obtain mean temperature on a monthly range. The original raster layer with a resolution of 1 km was downscaled to a finer resolution of 100 m with bilinear interpolation. Other covariates utilized in Chapter III (i.e., flower richness) were retrieved directly in the field through visual survey by recording the number of species during random inspection walks.

DNA metabarcoding

To characterize the taxonomic composition of pollen pellets removed from bumblebees' corbiculae in the study presented in Chapter III, we adopted a single-marker DNA metabarcoding approach.

Traditional methods for pollen identification rely on morphological analyses, which can be time-consuming and demands a high level of expertise. In contrast, modern investigative techniques based on genetic analysis, such as DNA metabarcoding, offer several advantages. These methods are rapid, do not require taxonomic expertise, and can offer a high level of taxonomical resolution (Chen et al., 2010;

Tommasi et al., 2021). DNA metabarcoding is akin to DNA barcoding but employs high-throughput sequencing technologies (HTS), enabling the simultaneous identification of multiple taxonomic units within a complex matrix. In the analysis conducted in Chapter III, pollen underwent DNA extraction by using Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), followed by the amplification of a specific DNA barcode region. The nuDNA ITS2 DNA barcode region was chosen for genetic analyses due to its characteristics, which include the availability of conserved regions for designing universal primers, the ease of amplification, its high discriminatory power (Chen et al., 2010), and its wide use in scientific studies also in the ecological context (Biella et al., 2022; Tommasi et al., 2022). Library preparation and sequencing (MiSeq 600 V3, 2 × 300-bp paired-end) were conducted at the Center for Translational Genomics and BioInformatics (San Raffaele Scientific Institute, Milan, Italy) and the obtained reads underwent comprehensive bioinformatic processing. Specifically, the metabarcoding data were quality-filtered and analyzed to obtain Exact Sequence Variants (ESVs) (Callahan, McMurdie & Holmes, 2017). The ESVs obtained were then taxonomically assigned through both automated and manually checked comparisons with reference sequences database (NCBI-GenBank) and the composition of each pollen mixture was then determined. Information obtained was used to evaluate pollen richness (i.e., the number of species found in each pollen sample), and the

community data were analyzed to investigate the putative correlation between species foraged and the nutritional features of the pollen pellet.

UV-vis and enzymatic assays

Multiple UV-vis-based assays were used to evaluate the chemical features of the sample analyzed in the different study-cases presented in the thesis. Specifically, such assays were employed for the determination of the overall polyphenol, flavonoid, total antioxidant content, carbohydrates, and starch composition. Generally, the reaction at the basis of these analyses depends on the ability of a particular chemical component (usually a dyer) to interact with some specific functional groups of the compounds/macromolecules of interest. Once the reaction is gained, the complex analyte-dyer is characterized by the absorption in a particular wavelength (usually in the visible light). Concerning the investigation of sugar components (i.e., free sugars in nectar or starch in seeds) we used enzymatic-based assays, exploiting the enzymatic reaction of the basic cellular metabolism, such as amidolysis, glycolysis, and the pentose phosphate pathway. All details on the assays performed and the enzymatic kit used are reported in the specific chapter.

HPLC quantitative analysis

Fluorescence detection (FLD) relies on the phenomenon where certain compounds when exposed to specific wavelengths of light (excitation wavelength), absorb the energy, and re-emit light at a longer wavelength (emission wavelength). This method is particularly advantageous for analyzing compounds with natural fluorescence, like many aromatic and conjugated molecules. Fluorescence detection offers enhanced sensitivity compared to UV detection, making it a preferred choice when dealing with trace-level analysis or complex matrices. The key difference lies in the way these techniques interact with the analytes: UV-vis detection relies on the absorption by analytes of light at specific wavelengths, while fluorescence detection utilizes the emission of light at a higher wavelength after excitation, allowing for highly sensitive and selective detection in HPLC-based analyses. Specifically, we used FLD detection for the analysis of amino acids content of nectar presented in Chapter I. Amino acids determination was performed using an automatic Pre-column-Derivatization RP-HPLC analysis. In detail, OPA and FMOC were used as derivatizing agents.

High-Resolution Mass Spectrometry

In various chapters of this thesis, a metabolomic approach based on the use of High-Resolution Mass Spectrometry (HRMS) was employed. Metabolomics refers to the

qualitative and quantitative analysis of naturally occurring small molecules. These molecules encompass substrates and products of cellular metabolism, such as sugars, organic acids, amino acids, vitamins, lipids, nucleotides, and phytochemicals. Metabolomics offers the advantage of being a cost-effective and rapid approach, facilitating the processing of large numbers of samples. Metabolism responds to environmental input and may therefore be used as a valuable tool to capture a snapshot of an organism's response to environmental stimuli or combinations of stimuli (Riedl et al., 2012). Metabolomic approaches are therefore acquiring importance in environmental and ecological studies. Specifically, the term “Ecological metabolomics” was first introduced by Macel et al., 2010 and indicates the application of metabolomics to ecological issues. In the studies presented in the thesis, an untargeted metabolomic approach was applied, that consists of the comprehensive analytical approach used to study the entire set of small molecules, or metabolites, within a biological sample. Unlike targeted metabolomics, where specific compounds are identified and quantified, untargeted metabolomics aims to capture a broad range of metabolites without prior knowledge of their identities. In an untargeted metabolomic approach, sophisticated analytical techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are employed to generate complex datasets containing information about the masses, retention times, and spectral

features of detected compounds. Advanced data analysis methods, including multivariate statistical analyses and bioinformatics tools, are then utilized to process and interpret these datasets. Researchers use untargeted metabolomics to explore metabolic pathways, discover biomarkers, and gain insights into the biochemical changes associated with various biological conditions such as diseases, environmental exposures, or genetic modifications. By providing a holistic view of the metabolome, untargeted metabolomics allows for the discovery of unexpected metabolites and patterns, making it a powerful tool in systems biology and biomedical research.

The study presented in this thesis focused on investigating the composition of phytochemicals endowed with nutraceutical properties, such as the antioxidant and anti-inflammatory activities exerted by foods in the human diet or the ability to prevent some stress phenomena for pollinator organisms, such as pesticide exposure, and parasites infections as functions of ecologically relevant variables.

To address these issues the samples underwent solid-to-liquid extraction procedures aimed at characterizing the phytochemical fractions (i.e., flavonoids, phenolic compounds, terpenes, alkaloids, etc.). The chromatographic condition that best addresses the investigation of this kind of analytes is the reverse phase chromatography, which foresees the exploitation of chromatographic columns characterized by a moderately hydrophobic stationary phase (such as the C-18 or

Byphenilic phase), where the analytes - which present hydrophobic moieties (e.g., aromatic rings, isoprene units, etc.) - tend to establish a certain interaction. The elution of these compounds is typically performed by exploiting chromatographic gradients that move from a polar condition (mainly based on an aqueous phase, usually coupled with formic acid) to an apolar solvent (mainly acetonitrile, or methanol in the case of Byphenilic columns) able to detach the analytes based on their polarity.

The detection of the analytes is roughly assessed by UV detection to better confirm the identity of a certain compound based on the ability to absorb in a particular light wavelength and then is effectively evaluated by exploiting High-Resolution Mass Spectrometry instruments. All analyses were conducted through Quadrupole Time-of-Flight (Q-ToF) mass spectrometry that operates based on two key principles: first, it uses a quadrupole mass analyzer to selectively filter ions based on their mass-to-charge ratio, allowing only ions of interest to pass through. These ions are then accelerated into a flight tube, where their time of flight is measured. The mass-to-charge ratio (m/z) and time of flight data are then used to determine the accurate mass of the ions, enabling highly precise identification and quantification of molecules in each sample. Once the raw data obtained through HRMS analyses were processed and statistical analysis conducted to determine if significant differences between samples exposed to differential

treatment/conditions occurred, the metabolites that were the most influential in determining these differences were identified based on MS^2 experiments (which encompassed different processes, ranging from untargeted analysis, such as Data Dependent Acquisition (DDA), Data Independent Acquisition (DIA/MS^e) to target MS/MS analyses for the identification of the fragments of specific ions). Specifically, for identification both literature references, public libraries (e.g., Natural Products Atlas) or proprietary library (Waters Traditional Medicine) were used. Details on sample preparation procedure, analysis parameters and statistical approaches applied are provided in the different chapters of the thesis.

Statistical analysis

The diverse nature of the data produced in this research project led to the use of a wide range of statistical methodologies tailored to the specific research question addressed in each Chapter. The analyses were performed by using multiple software. The processing of the metabolomic data were performed in MS-DIAL ver 5.1.23 and UNIFI ver 1.9.4.053 while all the other statistical analyses were conducted by using R ver 4.3.1 (R Core Team). All the data produced are shared on public repositories (details on the repositories used are reported in each Chapter).

Data produced were analyzed through different statistical pipelines. Both linear and generalized linear regression models were used in the different chapters of the

thesis. In each case, the type of data to be analyzed was considered and proper distribution was then selected. Random effects and interaction terms have also been included when the experimental design requires them.

Metabolomic and genomic data were also analyzed through multivariate statistical approaches such as Non-Metric Multidimensional Scaling (NMDS), Response Surface Model (RSM), Principal Component Analysis (PCA), and Redundancy Analysis (RDA) that are suited for the analyses of community data such as those represented by the specific composition of pollen mixtures or the metabolomic profile of floral resources and foods.

All the statistical approaches used are presented in detail for each study case in the respective chapter.

Synopsis

The thesis comprises four chapters, each one addressing a key aspect of pollination ecology and including the relevant publication produced.

The first chapter include three case studies. Case study I and II deals with the methodological approach for pollen and nectar sampling. The first case study introduces an innovative tool for pollen sampling, the Electronic Pollen Sampler (E-PoSa) that has been developed during this thesis project. The publication describes the assembly procedure and use of the new tool. It also compares the recovery rate and specificity of E-PoSa with other traditional techniques commonly used in ecological studies to sample pollen from flowers. The data obtained indicated E-PoSA as a viable alternative to traditional methods due to its significantly higher recovery capacity and precise sampling, minimizing the risk of contamination by other floral tissue. The second case study presents a publication that extends the investigation of the previous one by comparing not only pollen sampling methodologies but also nectar sampling techniques. Additionally, the comparison was conducted by looking not only at the macronutritional composition of the sample obtained but also at the metabolomic profile, specifically phytochemicals, characterized by means of HRMS. This study clarified that the sampling techniques may bias the chemical profiling of flower resources, which in turn may impact the

interpretation of the nutritional ecology and foraging patterns across landscapes and studied species. Overall, these two case studies achieves the goal of responding to a diffused concern about the comparability among studies related to the chemical characterization of pollen and nectar, given the significant heterogeneity methods found in the literature. Acknowledging the potential influences of the sampling techniques and moving towards shared standardized field protocols will advance the comprehension of species interactions and foraging patterns of pollinators and their nutritional needs. Furthermore, the new device presented is a cheap and easy-to-assemble tool encouraging its future use not only in the field of pollen nutrition but also in a wide variety of other contexts related to pollination ecology, botany, and breeding programmes. The tool developed was used in Case study II, III, and V.

The third case study answers the question whether land use and UHI can induce variations in pollen and nectar chemistry. The chemical composition of these rewards and their intraspecific variations were investigated by examining seven local wild plants commonly visited by pollinators within varying urbanization and agricultural intensity gradients and considering also local temperatures as a covariate. The main results indicated a significant impact of land use on pollen and nectar nutritional composition. Specifically, agricultural areas were associated with diminished sugar content and increased antioxidant activities in nectar and pollen

respectively, while urbanization was associated with an increase in the flavonoid concentration in pollen. These effects varied in a species-specific manner, as revealed also through untargeted metabolomic analyses. These observations provide essential insights into pollinators' nutritional ecology in anthropized environments and into the possible variation in the quality of their diet, a topic further explored in the next chapter (Case study IV). These emphasize the need for interventions aimed at guaranteeing the access of pollinators to high-quality food resources, paving the way of designing nutritionally adequate Nature based Solutions (NbS) for the management of green spaces in human altered environments.

In the second chapter of the thesis, the intricate interplay between habitat fragmentation, floral resources, and bumblebee foraging dynamics and diet quality is investigated. The study used urban green areas as dynamic open-air laboratories, offering stark contrasts for investigation. The primary aim was to unravel how fragmentation and local flower richness influence the bumblebee diet. The investigation encompasses diverse facets, including the nutritional content and plant composition of collected pollen pellets, foraging rates, and the associations between plants foraged and nutrition along a fragmentation gradient. The findings revealed a negative relationship between nutritional quality and habitat fragmentation, indicating compromised resource accessibility. Tight plant

composition-nutrition associations, suggesting limited access to alternative resources, was also observed. Furthermore, longer foraging trips in smaller green areas, highlighting behavioral constraints dictated by landscape features were registered. This research illuminates the intricate connection between landscape features and the nutritional ecology of pollinators. Importantly, these findings can offer valuable guidelines for policymakers and stakeholders engaged in the management and ecological restoration planning of urban green spaces and can inform the delineation of effective mitigation measures within urban contexts.

The third chapter looks at the relationship between animal pollination and food security through an experimental study that investigated both parameters related to the commercial quality and productivity and the metabolomic variations in *F. vesca* "fruits" and *V. unguiculata* seeds produced by flowers exposed to different pollination treatments, with putative consequences for human nutrition. The two species were selected to represent crops showing different reliance on pollination for fruit set and reproduction and to investigate phylogenetically distant species. The study was conducted in collaboration with the CREA institute of Sanremo, where wild strawberries were cultivated in open greenhouses and cowpea plants were cultivated in the open field. Flowers underwent differential pollination treatment that included forced self-pollination, hand-pollination (cross-pollination), and open pollination. Both morphological and chemical analyses were

carried out on the fruits and seeds. The results indicated that the pollination treatments not only determined variations in the morphology of the produced seeds and fruits but also played a significant role in shaping their phytochemical composition. This study is the first to look at the impact of pollination on the phytochemical profile of food and opens a new horizon in the recognition of the crucial role played by pollinators in guaranteeing food security and human well-being.

Part II

Publications

Chapter I

Impact of land use and related stressors on pollen and nectar chemistry

Case study I. Pioltelli, E., Guzzetti, L., Tonietti, L., Copetta, A., Biella, P., Campone, L., & Galimberti, A. (2023). E-PoSa: a novel and effective tool for sampling pollen directly from flowers. 2023-06. DOI: 10.1111/2041-210X.14241.

Type of article: Practical tools article

Status: Accepted in "*Methods in ecology and evolution"*

E-PoSa: a novel and effective tool for sampling pollen directly from flowers

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KEYWORDS

E-PoSa- Nutritional ecology - Pollen - Pollinators diet - Plant-pollinator interaction

AUTHOR CONTRIBUTIONS

Emiliano Pioltelli, Luca Campone, Luca Tonietti and Andrea Galimberti conceived the ideas and designed the methodology; Emiliano Pioltelli, Lorenzo Guzzetti and Andrea Copetta collected the data; Emiliano Pioltelli and Lorenzo Guzzetti analyzed the data; Emiliano Pioltelli, Lorenzo Guzzetti, Paolo Biella, and Andrea Galimberti led the writing of the manuscript. All authors critically contributed to the draft and gave their final approval for publication.

DATA AVAILABILITY

All data can be accessed at the following FigShare link:

https://doi.org/10.6084/m9.figshare.24291169.v1

Abstract

1. Pollinator insects are declining worldwide, also due to the alteration of their diet with severe implications on their health status. Pollinators diet relies mainly on flower rewards (i.e., pollen and nectar) and a precise characterization of their chemical composition is crucial in defining pollinators' nutritional ecology. In this context, the pollen represents a challenging source to investigate, especially due to operative challenges during collection operations and to the small amounts produced per flower.
2. Here, we designed and tested a novel, easy-to-assemble tool for pollen sampling: E-PoSa (Electronic Pollen Sampler), based on the use of a portable vacuum cleaner. We compared it with some of the most used sampling methods for pollen (i.e., anthers sieving and sampling of the whole anthers) by looking at the differences in their quantitative recovery and nutritional profile. Its applicability in ecological studies was also corroborated by an assessment of its recovery rate obtained from a panel of wildflower species in an operational environment.
3. The data obtained showed a significantly higher pollen recovery capacity of E-PoSa compared to the conventional sieving approach and the success in retrieving enough pollen to conduct phytochemical analyses from a broad range of flower morphologies in the field. Our results also demonstrated that high

purity pollen can be collected with E-PoSa and that the device does not introduce any significant variation in the nutritional analysis compared to the conventional sieving.

4. This new sampling approach represents a cheap and easy-to-assemble tool encouraging its future use not only in the field of pollen nutrition but also in a wide variety of other contexts related to pollination ecology. Acknowledging the potential influences of the sampling techniques and moving towards shared standardized field protocols will advance the comprehension of species interactions and foraging patterns of pollinators and their nutritional needs.

Introduction

Insect pollinators are declining worldwide due to multiple global issues such as climate change (Vasiliev D. & Greenwood S., 2021), exposure to pesticides (Goulson et al., 2015), and habitat loss (Potts et al., 2016). The depletion of dietary resources (i.e., pollen and nectar) due to the loss of flower-rich habitats and/or their contamination due to the use of agrochemicals is one of the main risk factors for pollinators (Vaudo et al., 2015; Hülsmann et al., 2015).

Considerable evidence about the importance of adequate nutrition for pollinators conservation have fostered a growing interest in the investigation of the nutritional landscape for a better understanding of the relationships existing between

pollinating insects and floral resources (Leonhardt et al., 2022; Vaudo et al., 2018; Vaudo et al., 2016; Venjakob et al., 2022). Many pollinators feed on pollen which represents the main protein source (Nicolson et al., 2018), and in this context, nutritional analyses of pollen are of paramount importance. However, these studies are often challenging at the analytical level, due to the scarcity of collected material or its frequent contamination by other floral parts (e.g., anthers and petals). Indeed, many flowers produce a low amount of pollen (< 1 mg; Jeannerod et al., 2022) and most of the research dealing with pollen nutrition focuses on the generally heavier pollen pellets collected by bees (Vaudo et al., 2018; Donkersley et al., 2017).

Besides studying pollen sampled from insect corbiculae, the evaluation of the nutritional composition of pollen directly collected from plants is relevant for the characterization of specific chemical features. Furthermore, as many environmental factors can shape plant metabolism (Ahmad et al., 2018), the isolation of pollen directly from different species may be an added value in investigating plant responses to environmental pressures and consequent effects on pollinators diet. On the application side, collecting and studying pollen can also elucidate which species are the most relevant to sustain the trophic demand of pollinators both in terms of quantity of pollen produced and in relation to its nutritional quality. However, to adequately carry out the wide array of chemical analyses required to quantify pollen nutritional composition (e.g., proteins, lipids, sugars, secondary

compounds, and amino acids), it is required to gain at least some quantity of pollen from each plant species, since each chemical component requires specific extraction methods and the analytical sensitivity of the chemometric approaches requires a significant amount of starting material, as well (Stabler et al., 2018).

To collect pollen, researchers have developed many sampling approaches to improve sampling efficiency and to face the critical issue of retrieving an adequate quantity of pollen to achieve reliable nutritional analyses (Jeannerod et al., 2022; Kendel and Zimmermann 2020; Roulston et al. 2005). However, many of these methods are time-consuming, as they require multiple steps of processing (e.g., 24 h drying, sieving) before obtaining the pollen samples that will be analysed (see also Table 1 for a comparison of advantages and drawbacks of the most common methods for pollen sampling). Furthermore, this wide panel of pollen sampling techniques results in a great heterogeneity among the different studies, hampering the comparison between the results obtained. With such a variety of available methodologies for the sampling of floral resources, the need to standardize the collection effort is becoming even more urgent.

Here, we propose a novel non-invasive tool for pollen sampling. The device, E-PoSa (Electronic Pollen Sampler), is based on the use of a commercially available portable vacuum specifically adapted to this purpose and allows the collection of highly pure pollen grains directly from the flower in a non-destructive way. To

validate this new approach, we provide a comparison with some of the most common methodologies adopted for pollen sampling to evaluate the magnitude of the differences that occur in the recovery efficiency and in the subsequent analysis of the nutrient composition.

Material and Methods

Assemblage and use of E-PoSa

To conduct a study on the pollen nutritional composition of wildflowers in northern Italy, we developed an effective and easy-to-assemble device that allows the collection of pollen with a yield suitable for chemical characterization. E-PoSa is based on the use of a portable vacuum cleaner with a removable plastic mask. The tool is made up of the following parts: a portable vacuum, a 5 mL tube with the head cut and the lid drilled; an inox mesh sheet, a paper filter, and laboratory film (Figure 1). The first step for assembling the device is preparing the 5 mL tube. The head of the tube needs to be cut approximately 0.5 - 1 cm apart from the tip using a sharp knife (Video S1). Second, the tube lid must be separated and drilled by using a conical drill bit (Video S1). The next step is cutting the paper filter with a diameter slightly larger than that of the test tube cap. Then, close the tube by paying attention that the paper filter remains in the correct position by completely covering the hole

previously made on the cap (Video S1). After cutting a small square from the inox mesh sheet, heat it quickly using a lighter, place it at the top of the tube, and hold it in place for a few seconds to ensure it does not detach (Video S1). The last step is to connect the tube to the head of the vacuum and secure it by using the laboratory film (Video S1). We suggest securing the tube to vacuum with multiple layers of laboratory film to avoid loosening during field sampling. The time required for the assembly is estimated to be less than 10 minutes. A few seconds are required for re-placing the pre-assembled collection tubes. The battery life of the portable vacuum used in this study is about 30 minutes, but it can be easily extended by integrating it with a portable power bank that allows its use also in remote sites. Details regarding the materials utilized and their costs are reported in Table S1.

The use of E-PoSa does not require expertise. The pollen can be collected by simply turning on the vacuum and moving it on the flowers to be sampled. Pollen is aspirated and accumulates on the paper filter. Due to the transparency of the tube, the amount of pollen collected can be easily estimated by eye and once enough pollen is collected, it can be directly transferred to another tube by removing the inox mesh from the tip of the E-PoSa and gently tapping on the bottom of the adapted tube. The system is useful with flowers of different morphology. In the case of small flowers or flowers that have anthers within the corolla (e.g., Lamiaceae or

Fabaceae) a 20 µL tip can be attached to the tube and fixed with laboratory film to allow a more accurate and effective pollen sampling.

Figure 1. Assemblage of the E-PoSa. Overview of all the materials needed for the assembly of the E-PoSa device (a. Portable vacuum; b. Paper filter with 25 µm pore size; c. 5 mL Eppendorf tube's cap drilled; d. 5 mL Eppendorf tube with Stainless steel mesh with 75 µm mesh size at the tip; e. strip of laboratory film); 2. Top view of the fully assembled E-PoSa 3. Frontal view of the fully assembled E-PoSa.

Study species

The target flower species were selected based on their taxonomy to account for a wider set of families characterized by different floral morphologies and different amounts of pollen produced. For the collection of anthers and pollen grains, a panel of three species was selected: *Tropaeolum majus* L. (Fam.: Tropaeolaceae), *Hippeastrum vittatum* Herb. (Fam.: Amaryllidaceae), *Alstroemeria aurea* Graham (Fam.: Alstroemeriaceae) (Fig. 2 A, B, C). The flowers were covered with a nylon mesh 24 h before sampling to avoid possible depletion of resources by pollinator visits. The study took place at the C.R.E.A Institute (Council for Agricultural Research

and Economics) of Sanremo, Italy where the studied species were cultivated in greenhouses.

Figure 2. Images of the flowers of all species studied. (A) *Tropaeolum majus* L. (Fam.: Tropaeolaceae); (B) *Hippeastrum vittatum* Herb. (Fam.: Amaryllidaceae); (C) *Alstroemeria aurea* Graham (Fam.: Alstroemeriaceae).

Pollen sampling

Three pollen collection approaches were adopted (Table 1; Fig. 3): i) anthers were collected by carefully removing them from the flowers using forceps; ii) pollen grains obtained by dehisced anthers through multiple steps of sieving starting with a mesh of 100 µm size to a final mesh of 50 µm in order to isolate pollen grains from other floral parts and used as control group (hereafter: mesh); (iii) E-PoSa developed in this study.

Figure 3. Photos of the different sampling techniques utilized for pollen collection: A. Removal of the anthers from *A. aurea* flower; B. Separation of pollen grains from the anthers of *H. vittatum* using a mesh; C. Pollen sampling with the E-PoSa device from *T. majus*.

For each sampling approach, we collected pollen from 25 flowers per species except for *H. vittatum*, for which only 15 flowers were sampled. The pollen obtained was pooled to gain enough material for all the subsequent analyses. All the collected samples were dried in an oven at 30°C for 12 hours. The three methods were compared for their recovery efficiency and the differences occurring in the macronutrient profiling. Details on the nutritional analysis are reported in Appendix A1.

Table 1. The table reports a brief description and the characteristics of the three methods used in this study for pollen sampling.

Method	Description	Volume/ Weight quantifiable	Operative environment	Destructive	Advantages	Constraints	Selected references
Anthers	Removal of the whole anthers from the flowers directly subjected to the extraction and analytical evaluation of nutrients.	No	*In situ*	Yes	Fast. It allows the sampling of a high amount of matrices and is applicable to all flower species.	Pollen grains are not isolated from the vegetative tissues of the anthers, making the chemical evaluation of pollen biased.	Kenđel and Zimmermann (2020); Arathi et al. (2018)
Mesh	Anthers are removed from the flowers and dried at 30 °C for 12- 24 h. Pollen grains are then isolated from the anthers through sieving using mesh with the desired size.	Yes	*Ex Situ*	Yes	The mesh size can be selected based on the specific size of the studied pollen grains. It allows a complete isolation of the pollen grains from other floral parts thus reducing contamination risk.	It requires a drying phase prior to the sieving process that can last up to 24 h. It is not performable directly on the field and the recovery is usually very low.	Jeannerod et al. (2022); Jacquemart et al. (2019)

E-PoSa	Pollen is vacuumed from the anthers with the assistance of a portable vacuum cleaner adapted with filters and collection tubes.	Yes	*In situ*	No	It allows the sampling and isolation of pollen grains directly on the field. It is a non-invasive method (i.e., it does not require to sample floral units and to bring them in the lab) which allows to collect pollen from many flowers in a short time and can be used in remote locations. Materials needed are cheap.	The use of this tool is limited by the battery capacity of the vacuum cleaner. This limit can be overcome by using a powerbank.	this study
Stem collection	Stems bearing flower units are cut and left in water overnight in the laboratory. The pollen is then shaken onto a flat surface and scraped into storage with a plastic ruler. The use of a tuning fork is suggested to retrieve pollen from poricidal anthers (and to a lower extent also from non-poricidal anthers). If the pollen becomes contaminated with other floral parts, a step of sifting through a sieve is required.	Yes	*Ex Situ*	Yes	The sampling procedure of the stems is fast and feasible with all plant species. If the sifting step is carried out, it allows a complete isolation of the pollen grains from ther floral parts thus reducing contamination risks.	The procedure is time consuming. Prior to separating the pollen from the mature anthers, the stems need to be maintained in the water overnight. A step of sifting through a sieve is also suggested to be sure that the pollen samples do not present any contamination by other floral parts, similarly to the "Mesh" technique.	Roulston et al. (2005)

Statistical analysis

The differences in the recovery of pollen (expressed as mg per flower) per each species were evaluated by using Generalized Linear Models (GLM) assuming a Gamma distribution of the response variable. The fixed effect was the sampling method. The software used was R (version 4.3.1) and the package used was “nlme” (Pinheiro & Bates, 2023) and “ggplot2” (Wickham, 2016). For details on the statistical analysis on pollen nutritional composition see Appendix A1.

Validation in operative environment

The E-PoSa was employed also in the operative environment to collect pollen from a diverse array of wildflowers in the framework of a project aiming at the characterization of the pollinators nutritional landscape of northern Italian meadows. The sampling protocol adopted was the same as in the greenhouse validation experiment. The number of flowers/inflorescences sampled per species was recorded in the field and related to the weight of the pollen retrieved to estimate both the pollen recovery per floral unit and the minimum number of floral units to be sampled to perform nutritional analysis.

Results

Compared to other sampling approaches, the adoption of the E-PoSa represents a cost-effective and easy-to-use tool for the sampling of pollen from a wide range of flower species with different flower morphology and pollen yields. As anthers sampling does not allow measuring the weight of the pollen retrieved directly, it was not possible to compare the mass recovered by this technique with the mesh and the E-PoSa. Pollen collection through the E-PoSa device resulted in a significantly higher amount of pollen retrieved in all the three analyzed species, as shown in Fig. 4. The present method let us perform a field sampling on at least 22 wildflower species belonging

to 8 families showing a broad range of flower morphology, that were successfully collected for the characterization of pollen nutrients (see Table 2 for details).

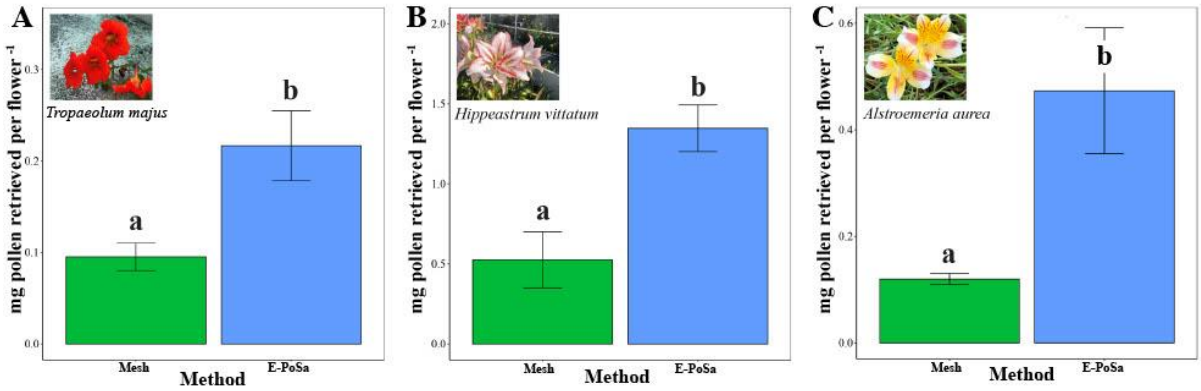

Figure 4. Barplots reporting the yield of pollen retrieved expressed as mg per flower depending on the sampling method (i.e., mesh and E-PoSa). (A) Data on *Tropaeolum majus*, (B) data on *Hippeastrum vittatum*, and (C) data on *Alstroemeria aurea*. For each of the species compared, $N = 2$ for mesh and $N = 3$ for E-PoSa. Values are the mean ± SEM. Significant differences ($p < 0.05$) are reported by different letters.

Pollen macronutrient composition

The estimation of the macronutrient composition of pollen is shown in Fig. 5 and Table S2. Generally, the results from all the three investigated species show that the sampling with the E-PoSa does not introduce any further bias than anthers and in most cases, it removes them, nearing the results of the nutritional investigation to those of mesh sampled pollen (Fig. 5a, 5b and 5c).

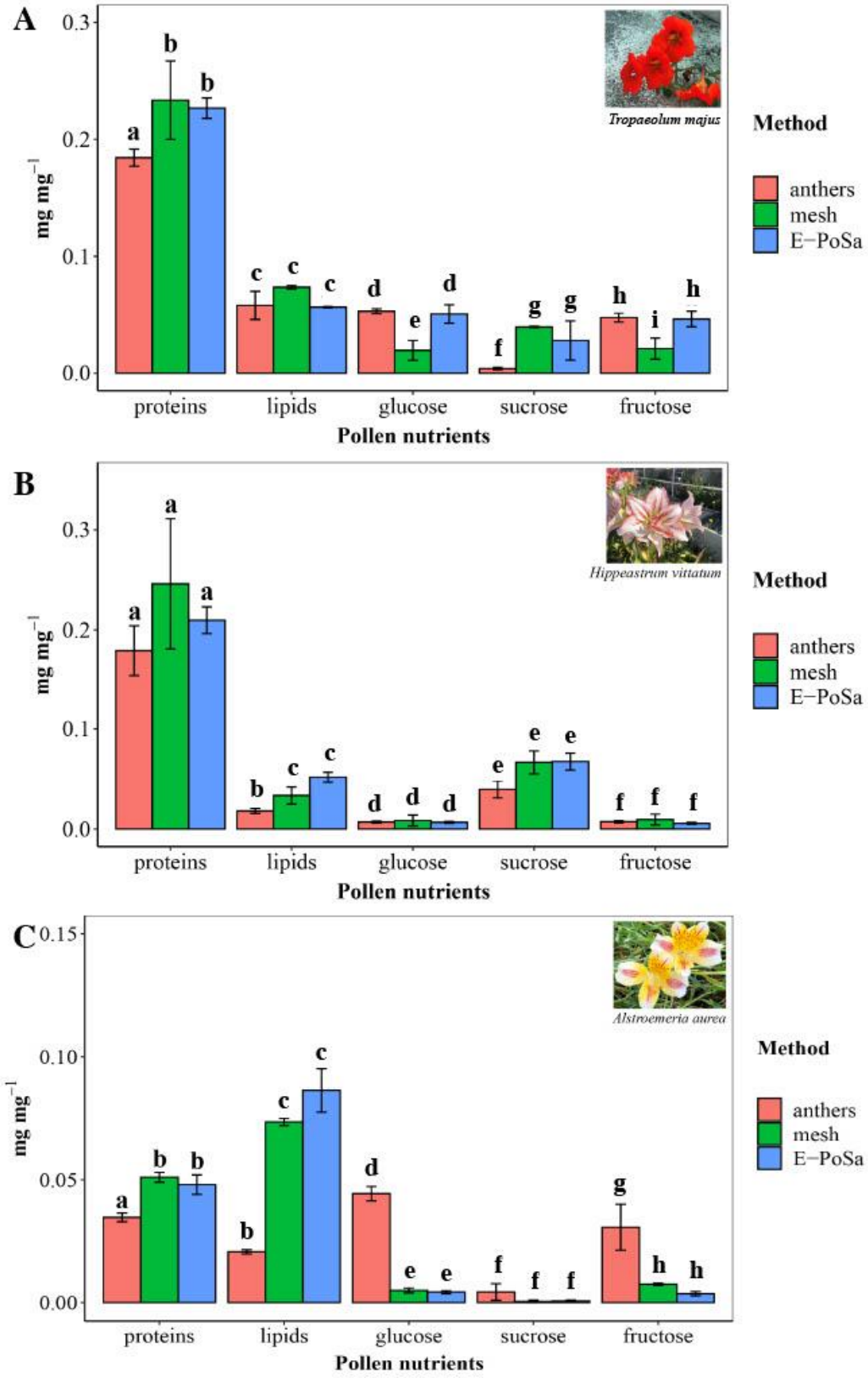

Figure 5. Quantified amounts of the macronutrient composition of the samples in the 3 species studied. Values are reported as the mean ± SEM. For each species, *N* = 3 for anthers, *N* = 2 for mesh, and *N* = 3 for E-PoSa samples. For each nutrient, significant differences ($p < 0.05$) among the three sampling methods are estimated through the post hoc Tukey test and are reported by different letters.

Table 2: The table provides information on pollen recovery from different wildflower species. It includes family, floral morphology, and the need for E-PoSa adaptation with the tip. The "minimum floral units" column indicates the required number of floral units for obtaining 2 mg of pollen, the minimum amount for macronutritional profiling (proteins, lipids, and carbohydrates) using the protocol described in Appendix A1.

Species	Family	Floral morphology	Floral shape	Need of tip adapter	Floral unit considered	µg / floral unit	Minimum floral units
Taraxacum officinale	Asteraceae		Composite flower	no	Inflorescence	196	11
Sonchus oleraceous	Asteraceae		Composite flower	no	Inflorescence	168	12
Hypochaeris radicata	Asteraceae		Composite flower	no	Inflorescence	133	16
Cyrsium vulgare	Asteraceae		Composite flower	no	Inflorescence	139	15
Crepis sp.	Asteraceae		Composite flower	no	Inflorescence	62	33
Bellis perennis	Asteraceae		Composite flower	no	Inflorescence	54	38
Erigeron annuus	Asteraceae		Composite flower	no	Inflorescence	37	55
Cichorium intybus	Asteraceae		Composite flower	no	Single flower	25	80
Lotus corniculatus	Fabaceae		Keel flower	yes	Single flower	89	23
Trifolium repens	Fabaceae		Keel flower	yes	Inflorescence	13	154
Trifolium pratense	Fabaceae		Keel flower	yes	Inflorescence	10	200
Salvia pratensis	Lamiaceae		Tubular flower with biradial symmetry	no	Single flower	36	56
Lamium purpureum	Lamiaceae		Tubular flower with biradial symmetry	yes	Single flower	29	69
Prunella vulgaris	Lamiaceae		Tubular flower with biradial symmetry	yes	Single flower	20	100

Malva sylvestris	Malvaceae		Bowl flower	no	Single flower	695	3
Oxalis acetosella	Oxalidaceae		Bowl flower	no	Single flower	18	112
Plantago lanceolata	Plantaginaceae		Blossom incospicuos	no	Inflorescence	230	9
Veronica persica	Plantaginaceae		Disk flower	yes	Single flower	19	106
Rubus sp.	Rosaceae		Bowl flower	no	Single flower	85	24
Prunus sp.	Rosaceae		Bowl flower	no	Single flower	72	28
Potentilla reptans	Rosaceae		Bowl flower	no	Single flower	67	30
Sambucus nigra	Viburnaceae		Brush blossom	no	Inflorescence	720	3

Discussion

The recent advances in pollination nutritional ecology require the definition of standardized sampling methods for pollen (Jeannerod et al., 2022). The analysis of pollen from wild plants needs to be fine-tuned for an accurate definition of its role in the nutritional balance of pollinators' diets (Lau et al., 2022). It is well known that the recovery of pollen grains from wild plants is a difficult task to perform since the anthers of entomogamous plant species usually produce low amounts of pollen (e.g., Jeannerod et al., 2022; Palmer-Young et al., 2019). Obtaining a significant amount of pollen without contamination originating from other floral parts can be very time-consuming, reducing the efficiency and the feasibility of nutritional ecology studies. The results of the nutritional analyses of pollen performed on the three target species show that this strategy avoids sample contaminations, since the pollen collected by the E-PoSa did not show any significant difference in terms of macronutrient composition compared to the pollen sampled by sieving. In this framework, we suggest the adoption of the E-PoSa for the non-invasive collection of a satisfactory amount of pollen in a relatively short time. The adoption of the E-PoSa does not require any expertise or training for its usage due to the ease of assembly and the getting of the equipment. Furthermore, it lets the sampling of pollen free of contaminants possibly impairing further nutritional analyses. This feature makes it possible to conceive its exploitation in multiple contexts, such as in the framework of citizen science activities, plant breeding programmes, and/or in contexts requiring the need to optimize the logistics and human resources available. Furthermore, we emphasize the significance of E-PoSa for studies dealing with pollen chemical defence. The swiftness of this sampling approach enables to capture a snapshot of the chemical profile in a specific moment, facilitating the investigation of short-term plant responses to experimental

treatments (e.g., herbivory, abiotic stressors). In addition, the E-PoSa sampling accuracy allows to obtain well-separated pollen samples, thus minimizing mischaracterizations linked to the considerable variations occurring in the chemical profile across different floral tissues and rewards (possibly reflecting their ecological roles). Moreover, the elimination of the drying step required by the sieving method would additionally be essential for the analysis of more labile components (e.g., antioxidants, and volatiles). At the same time, we underline that steel mesh, Eppendorf tubes, and filter paper need to be prepared before each sampling, especially when pollen from different plant species is sampled. This might be particularly important when chemical analyses are carried out and chemical contaminations among the pollen of different plant species should be avoided.

Conclusions

The field of nutritional ecology in the context of plant-pollinator interactions is growing in importance to address the issues of pollinator safeguarding and conservation. The novel E-PoSa can provide a cheap and easy-to-assemble tool, encouraging its future use in the field of pollen research, including breeding, interactions with herbivores, and ecological studies dealing with the characterization of the nutritional landscapes for pollinators. The effectiveness of the recovery allows the sampling to be performed on a wide range of species considering the availability of floral resources usually occurring in meadows (see Table 2) with a high degree of purity, as proved by the nutritional characterization of samples.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest.

ACKNOWLEDGEMENTS

Funder: Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of the 18 December 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU.

Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP, H43C22000530001 Project title “National Biodiversity Future Centre - NBFC”.

Luca Tonietti is supported by the PhD Program PON “Ricerca e Innovazione” 2014-2020, DM n. 1061 (10/08/2021) and n. 1233 (30/07/2020).

The authors are grateful to Barbara Ruffoni, Paolo Mussano and the team of the CREA of Sanremo for their support. A special thank goes to Fausto Ramazzotti for his help during the sampling activity.

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Appendix A1

TEXT S1

POLLEN NUTRITIONAL ANALYSIS

Prior to the nutritional analyses, all the pollen samples were freeze-dried to normalize each one to the dry weight. All the nutritional analyses were performed on ≅ 1 mg for at least three biological replicates per method per species. Macronutrients (i.e., proteins, lipids, and sugars) were extracted by following the protocol proposed by Vaudo et al., 2016 with minor modifications.

Extraction and quantification of protein content

To analyze the protein concentration of pollen loads we performed the Bradford assay. For each sample, approximately 1 mg of pollen was weighed in triplicate and added to a volume of 300 µL 0.1 M NaOH solution. To allow the mechanical lysis of the pollen wall, the samples were subjected to 3 cycles of direct sonication lasting 10 seconds each by an ultrasound probe sonicator (Branson, USA). At the end of the process, 1.2 mL 0.1 M NaOH was added, and the samples were maintained at 4°C for 24 h to allow the chemical lysis and then centrifuged at 14,000 x g for 5 minutes. The extracts were tested performing the Bradford assay using the Coomassie brilliant blue dyer (ThermoScientific, USA) and the bovine serum albumin or BSA (Merck, Germany) as analytical standard (range 0-1500 µg/mL). Samples absorbance was measured at a wavelength of 595 nm against the blank. The entire process was performed by keeping the samples on ice to avoid possible protein degradation phenomena.

Extraction and quantification of lipid and carbohydrates content

For the extraction of lipids and carbohydrates, a volume of 200 µL of Na_2SO_4 2% w/v was added to 1 mg of pollen (in triplicate for each sample) and then the mechanical lysis was performed by probe ultrasonication (Branson, Digital Sonifier, USA). Then samples were transferred to a glass tube and 1.6 mL of a 1:1 v/v $CHCl_3$/MeOH solution was added. The tubes were vortexed and centrifuged for 5 min at 3,200 x g. The supernatant was transferred to a clean glass tube, then 600 µL of ultrapure Milli-Q H_2O was added and the samples were centrifuged at 3,200 x g for 5 minutes. This process led to forming of two clearly distinguishable phases: the upper one composed of MeOH and H_2O containing carbohydrates and polar molecules, and the underlying one made of $CHCl_3$, containing lipids. The two phases were separated and the whole process was repeated twice to maximize the extraction efficiency of the analytes of interest.

For the quantification of lipids, we conducted the Vanillin assay using soybean vegetable oil as standard (range 5.75-920 µg). The samples were soaked at 60°C until complete evaporation of $CHCl_3$. Hereafter, 200 µL H_2SO_4 98% v/v solution was added, and the temperature was raised up to 100°C. After 10 min, 4.8 mL of vanillin (1.2 mg/mL in 68% v/v H_3PO_4) was added. Samples were read against the blank at the wavelength of 490 nm.

The content of free sugars was carried out by using a commercial enzymatic kit (Megazyme, Ireland) as described in Biella et al., 2022 and total sugar composition was evaluated as the sum of three free sugars (i.e., sucrose, glucose, fructose).

Statistical analysis

To test for differences in the estimation of the macronutrient in the pollen we set a series of Generalized Linear Mixed Effects Models (GLMMs) with a binomial/beta-binomial distribution of the dependent variable. The sampling method was treated as a fixed effect while the species were included as random factors in R (version 4.2). Packages exploited were *"glmmTMB", "plyr", "ggplot2", "MuMIn",* and *"multcmp"*. *P*-values for multiple comparisons were adjusted by using Tukey post hoc test for multiple comparisons.

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Vaudo, A. D., Patch, H. M., Mortensen, D. A., Tooker, J. F., & Grozinger, C. M. (2016). Macronutrient ratios in pollen shape bumble bee (Bombus impatiens) foraging strategies and floral preferences. *Proceedings of the National Academy of Sciences*, *113*(28), E4035–E4042. https://doi.org/10.1073/pnas.160610111

Supplementary information

Table S1. Price of the necessary hardware for E-PoSa assembly

Item	Provider	Cost (€)	Note
Mini-USB Vacuum Cleaner	Honk (Amazon)	22.99	
Inox mesh 30 x 100 cm. 0,075 mm aperture	TIMESETL (Amazon)	16.99	
Power Bank 26800mAh, Portable charger USB C Powerbank Battery	Charmast (Amazon)	34.99	Optional

Table S2. Quantified amounts of mg pollen retrieved per flower unit and mg of nutrients per mg of pollen. Values are the mean ± SEM (see Figure 4 and 5 for further details).

Species	Method	*n*	Yield (mg * flower^{-1})	Proteins (mg * mg pollen-^{1})	Lipids (mg * mg pollen-^{1})	Glucose (mg * mg pollen-^{1})	Sucrose (mg * mg pollen-^{1})	Fructose (mg * mg pollen-^{1})
Tropaeolum majus	anthers	3	-	0.184 ± 0.007	0.058 ± 0.012	0.053 ± 0.003	0.004 ± 0.001	0.048 ± 0.004
	mesh	2	0.095 ± 0.015	0.234 ± 0.034	0.038 ± 0.006	0.020 ± 0.009	0.040 ± 0.001	0.021 ± 0.009
	E-PoSa	3	0.217 ± 0.038	0.227 ± 0.009	0.057 ± 0.001	0.051 ± 0.008	0.028 ± 0.017	0.046 ± 0.007
Hippeastrum vittatum	anthers	3	-	0.179 ± 0.025	0.018 ± 0.003	0.007 ± 0.001	0.040 ± 0.009	0.007 ± 0.001
	mesh	2	0.525 ± 0.175	0. 246 ± 0.065	0.034 ± 0.009	0.009 ± 0.006	0.067 ± 0.012	0.010 ± 0.006
	E-PoSa	3	1.347 ± 0.147	0.210 ± 0.013	0.052 ± 0.005	0.007 ± 0.001	0.067 ± 0.009	0.006 ± 0.001
Alstroemeria aurea	anthers	3	-	0.035 ± 0.002	0.021 ± 0.001	0.044 ± 0.003	0.004 ± 0.003	0.031 ± 0.009
	mesh	2	0.12 ± 0.01	0.051 ± 0.002	0.074 ± 0.002	0.005 ± 0.001	0.001 ± 0.001	0.008 ± 0.001
	E-PoSa	3	0.477 ± 0.118	0.048 ± 0.002	0.086 ± 0.001	0.004 ± 0.001	0.001 ± 0.000	0.004 ± 0.001

Video S1. Link for the video demonstrating E-PoSa assembly and field application.

https://youtu.be/Rt4KCrGGwlE?si=H_PAWyOdQDbldy9w

Case study II. Pioltelli, E., Guzzetti, L., Copetta, A., Labra M., Galimberti, A., Guidi Nissim W., Biella, P. (2023). Assessing the analytical reliability of traditional and novel sampling methods for the study of flower rewards 2023-06. DOI: 10.1111/2041-210X.14241.

Type of article: Methods, Tools, and Technologies

Status: Under review in "*Ecosphere*"

Assessing the analytical reliability of traditional and novel sampling methods for the study of flower rewards

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KEYWORDS

HRMS - Nutritional ecology - Phytochemicals - Plant-pollinator interaction - Pollen and Nectar - Pollinators diet

OPEN RESEARCH STATEMENT

The corresponding author ensures that all data will be made available in the Figshare repository upon acceptance.

Abstract

Pollinator insects are declining worldwide also due to the alteration of their diet which plays a pivotal role in influencing their health status. Despite interspecific and intraspecific diversity in the diet, pollinators share a complete or partial reliance on pollen and nectar as food sources, for example in bees and flies, respectively. A precise characterization of the chemical composition of these flower resources represents a key step in the definition of pollinators' nutritional ecology. In the past decades, pollen and nectar represented challenging sources to investigate. The main challenge for the sampling of pollen and nectar is their small amounts per flower, making them difficult to collect.

Here, we compared a recently proposed tool based on a portable vacuum cleaner (E-PoSa, i.e., Electronic Pollen Sampler) with some of the most used sampling methods for pollen (i.e., anthers sieving and sampling) and studied nectar sampling techniques (i.e., centrifugation, microcapillaries, washing, and micro-rinsing) by looking at the differences in their quantitative recovery as well as their nutritional profiling. Pollen and nectar were collected from three model flower species each.

Our results demonstrated that different collection methods introduce biases in the nutritional profiling of floral rewards with variations in their qualitative and quantitative composition. The quantification of the nutrient composition (i.e., sugars in nectar and proteins, sugars, and lipids in pollen) showed under- or

overestimation depending on the methodology utilized for the sampling. Similar outputs were obtained by looking at the phytochemical composition and by considering the sampling efficiency. Anthers sampling was associated with inaccurate chemical profiling compared with the E-PoSa-based sampling which showed an accuracy comparable to conventional pollen sieving.

This study clarifies that the sampling techniques may bias the nutritional profiling of flower resources, which in turn may impact the interpretation of the nutritional ecology and foraging patterns across landscapes and studied species. Acknowledging the potential influences of the sampling techniques and moving towards shared standardized field protocols will advance the comprehension of species interactions and foraging patterns and nutritional needs of pollinators.

Introduction

Insect pollinators are declining worldwide due to multiple global issues (Cardoso et al., 2020) such as climate change (Vasiliev D. & Greenwood S., 2021), exposure to pesticides (Goulson et al., 2015), and habitat loss (Potts et al., 2016). However, another main threat is represented by the reduction of food resources available with consequent impoverishment of the pollinators diet, both in terms of quantity and quality of foraged pollen and nectar (Jones & Rader, 2022; Vaudo et al., 2015; Hülsmann et al., 2015), with implications also for plant-insect interactions (Jamieson et al., 2017). Land-use intensification related to the

expansion of urban areas and agricultural intensification is significantly reducing the extension and connectivity of suitable habitats and trophic resources for pollinators (Lau et al., 2023; Wenzel et al., 2020;). Moreover, progressive (e.g., due to climate change events) or sudden (e.g., due to local scale transformation of habitats) changes in environmental conditions such as temperature, light exposure, and precipitation influence plant physiology, thus altering the chemical composition of their floral resources (Biella et al., 2022; Russell & McFrederick, 2022).

Pollen and nectar display relevant differences in their chemical composition (Palmer-Young et al., 2019) with implications both on the nutritional and ecological perspective. Different compounds found in pollen and nectar can mediate ecological interactions, such as pollinators attraction to flowers (Galen et al., 2011; Junker et al., 2010), the inhibition of microbial activities (Junker & Tholl 2013), and chemical defense preventing excessive pollen harvesting (Vanderplanck et al., 2020). If considering macronutrient composition, nectar is rich in free sugars and provides ready-to-use energy (Nicolson et al., 2018) representing the main carbohydrate source for adult bees (Pamminger et al., 2019), whereas pollen has a high protein content and at least in bees, it represents the main food source for larvae (Nicolson, 2011). Multiple evidence about the importance of adequate nutrition for pollinators conservation has fostered a growing interest in the investigation of the nutritional landscape for a better understanding of the relationships existing between pollinating insects and floral resources (Leonhardt

et al., 2022; Jamieson et al 2017; Vaudo et al., 2018; Vaudo et al., 2016; Venjakob et al., 2022). In this context, nutritional analyses of pollen and nectar are of paramount importance. However, these studies are often challenging due to the scarcity of nectar and pollen whose sampling is often subjected to contamination by other floral parts (e.g., anthers and petal parts). Indeed, many flowers produce a low volume of nectar (< 1 µL; Power et al., 2018) and a low amount of pollen (< 1 mg; Jeannerod et al., 2022).

To improve the sampling efficiency and to face the critical issues of the retrieval of an adequate quantity of pollen and nectar for achieving reliable nutritional analyses, researchers have developed many sampling approaches for floral rewards (Pioltelli et al., 2023). This wide panel of techniques for pollen and nectar sampling results in a great heterogeneity among the different studies, hampering the comparison among the results obtained, as also claimed by Power et al, 2018 and Morrant et al., 2009. Such limitations are a concern since this kind of data carries significant implications for comprehending the overall status of pollinators. Indeed, besides macronutrients (i.e., proteins, sugars, and lipids) which have a primary role in the development, sustenance, and nutrient regulation of pollinators (Vaudo et al., 2016; Nicolson, 2011; Di Pasquale et al., 2013), pollen and nectar also represent a source of micronutrients. They contain vitamins, minerals, phytosterols, and amino acids that play crucial roles in diverse biological processes

(Lau et al., 2023), from larval development (Vanderplanck et al., 2014) to learning performance (Palmer-Young et al., 2019). Another class of relevant compounds is phytochemicals (i.e., flavonoids, phenolic acids, terpenes, and alkaloids), which are even more considered for the role they may play for pollinators at the physiological level (Niño et al., 2022; Ardalani et al., 2021; Koch et al., 2019; Mao et al., 2013). Pollen and nectar contain secondary compounds able to influence pollinators' health through the reduction, prevention, or by increasing tolerance to infections (Koch et al., 2017; Richardson et al., 2015), and can also play an important role in coping with oxidative stress (Berenbaum & Calla, 2021), thus acting as nutraceuticals in pollinators diet (Stevenson et al., 2022; Ardalani et al., 2021). Despite the potential beneficial effects on pollinators health, they can display some toxicity or act as a deterrent, often in a dose-dependent manner (Vanderplanck et al., 2020). Furthermore, the chemistry of floral rewards contributes to the definition of plant-pollinator interactions and of the collection specificity to plant taxa (Ruedenauer et al., 2020; Woodcock et al., 2014). These issues require the sampling effort to be performed with the assurance of both precise sampling of the matrices and in enough quantities to be chemically characterized. With such a variety of methodologies for the sampling of floral resources, each one with different benefits and drawbacks (Pioltelli et al., 2023), the need to standardize the collection effort is becoming even more urgent.

Here, we provide a comparison of some of the most common methodologies adopted for both pollen and nectar sampling to evaluate the magnitude of the differences occurring in the analysis of macronutrients and secondary compounds. Therefore, the aims of the present study are: i) to compare different protocols for the sampling of flower resources to assess their reliability in terms of recovery and nutritional profiling; ii) to understand the magnitude of the biases generated by non-specific sampling and how to cope with these issues.

Material and methods

Study species

The target flower species were selected based on their taxonomy to account for a wider set of families characterized by different floral morphologies and different amounts of pollen and nectar produced. For the collection of anthers and pollen grains, a panel of three species was selected: *Tropaeolum majus* L. (Fam.: Tropaeolaceae), *Hippeastrum vittatum* Herb. (Fam.: Amaryllidaceae), *Alstroemeria aurea* Graham (Fam.: Alstroemeriaceae) (Fig. 1 a, b, c). Nectar sampling was performed on 3 nectariferous species: *Agapanthus praecox* FM. Leight (Fam.: Amarillidaceae), *Russelia equisetiformis* Schletcht. & Cam. (Fam.: Scrophulariaceae) and *Salvia greggii* A. Grey var. 'Purple Queen' (Fam.: Lamiaceae) (Fig. 1 d, e, f). For each species, pollen and nectar were collected from the flowers beard by the same

plant, to minimize variations due to the genetics of the individuals. Sampled flowers were covered with a nylon mesh 24 h prior to the sampling to avoid possible depletion of the resources by pollinator visits. The study took place at the C.R.E.A Institute (Council for Agricultural Research and Economics) of Sanremo, Italy where the studied species were cultivated in greenhouses.

Figure 1. Images of the flowers of all the studied species. (a) *Tropaeolum majus* L. (Fam.: Tropaeolaceae); (b) *Hippeastrum vittatum* Herb. (Fam.: Amaryllidaceae); (c) *Alstroemeria aurea* Graham (Fam.: Alstroemeriaceae); (d) *Agapanthus praecox* FM. Leight (Fam.: Amarillidaceae); (e) *Russelia equisetiformis* Schletcht. & Cam. (Fam.: Scrophulariaceae); (f) *Salvia greggii* A. Grey var. 'Purple Queen' (Fam.: Lamiaceae).

Pollen sampling

Three pollen collection approaches were adopted: i) anthers were collected by carefully removing them from the flowers using forceps; ii) pollen grains obtained

by dehisced anthers through multiple steps of sieving starting with a mesh of 100 µm size to a final mesh of 50 µm in order to isolate pollen grains from other floral parts and used as the control group (hereafter: mesh); (iii) E-PoSa (Pioltelli et al., 2023), by vacuuming the pollen from each flower for 10 seconds (details on the tool are reported in Text S1 and Fig. S1). For each sampling approach, we collected pollen from 20 flowers per species to gain enough material for all the subsequent analyses. All the collected samples were dried in an oven at 30°C for 12 hours.

Nectar sampling

Nectar was sampled by following 4 different protocols: i) nectar was sampled by washing the flowers in 15 mL tubes containing 2 mL H_2O for 30 seconds per flower hereafter: wash; ii) nectar was sampled by adding from 2 to 20 µL of H_2O (depending on the nectar viscosity) and then recovered by using glass syringes (Hamilton, USA) - hereafter: microrinsing; i) nectar was sampled by using commercially available glass capillary (Merck, Germany) - hereafter: capillary; iv) nectar was sampled by centrifuging flowers in a 50 mL tube endowed with a wire mesh (20 µm diameter) and then recovered with a pipette at the bottom of the tube - hereafter: centrifuge. Nectar collection was performed between the 25th and 26th July 2021 in an hour range between 10 am and 12 am for all the species to minimize as much as possible biases due to the daytime. Samples were stored in a 1:1 v/v nectar/EtOH ratio to

avoid microbial-mediated degradation of the occurring compounds and stored at -80°C up to the analyses.

Chemical characterization of pollen samples

Prior to the nutritional analyses, all the pollen samples were freeze-dried to normalize each one to the dry weight. All the nutritional analyses were performed on ≅ 1 mg for at least three biological replicates per method per species.

Macronutrients (i.e., proteins, lipids, and sugars) were extracted by following the protocol proposed by Vaudo et al., 2016 with a minor modification for the extraction of lipids which was carried out with two subsequent extraction cycles. The secondary compounds were extracted by a hydro-alcoholic solvent made of EtOH/H_2O 1/1 v/v in a drug/solvent ratio of 1:1000 w/v for 3 cycles of extraction in an ultrasound bath (37 Hz, 30°C). Samples were dried under gaseous nitrogen and resuspended in 1 mL ultrapure Milli-Q H_2O. The total nutrient composition of samples was evaluated by using different colorimetric assays. In particular, the Bradford method was used for protein quantification and the vanillin assay for the detection of lipids. The content of free sugars was carried out by using a commercial enzymatic kit (Megazyme, Ireland). For the analysis of sucrose 100 µL of β-fructosidase are added to 50 µL of sample (or H_2O for the blank) followed by an incubation of 5 min. Consequently, the analysis of free sugars (glucose and fructose)

starts by mixing 50 µL of sample/H_2O to 1050 µL of H_2O, while for the sucrose analysis 950 µL H_2O are added. Then, for both analyses, we added 50 µL buffer pH 7.6 and 50 µL of a solution $NADP^+$/ATP followed by an incubation of 3 mins.

At this point, the absorbance (A_1) is read at 340 nm to monitor the basal level of each sample (against the blank). Then the analysis foresees the addition of 10 µL of the enzymes hexokinase and glucose 6-phosphate dehydrogenase followed by an incubation of 5 mins. Then the absorbance is read at 340 nm (A_2). The difference value between A_2 and A_1 refers to the glucose content. Finally, 10 µL of phosphoglucose isomerase are added and each sample is incubated for 10 mins at room temperature. Then the absorbance is read again at 340 nm (A_3). The difference value between A_3 and A_1 refers to the fructose content. The content of sucrose is obtained by the difference between the absorbances of samples incubated without and with the enzyme β-fructosidase. The total phenol content, total antioxidant activity and total flavonoid content was evaluated by exploiting the protocols suggested in Unpublished manuscript Pioltelli et al.b and Unpublished manuscript Pioltelli et al.c. The hydro alcoholic extracts were ten-fold diluted and analysed through RP-LC-HRMS (See Text S2 for details).

Chemical characterization of nectar samples

Nectars were dried under nitrogen and resuspended in 1 mL ultrapure Milli-Q H_2O. The solution was filtered using hydrophilic polytetrafluoroethylene (PTFE) filters

with 0.22 µm pore size (Macherey-Nagel, Germany) to obtain a clean solution. Samples were analysed for the free sugars content and phytochemicals profile as reported for pollen samples.

Statistical analyses

To test whether the sampling method affects the amount of nectar retrieved and the nutrient composition of floral rewards, we set a series of Generalized Linear Mixed Effects Models (GLMMs) with a binomial or beta-binomial distribution of the dependent variable for pollen nutritional analysis depending on the overdispersion parameter (if higher than 1 a beta-binomial distributed models were run to avoid type I errors), gaussian or Gamma distribution (depending on data normality) for nectar analyses. The sampling method was treated as a fixed effect while the species were included as random factors in R (version 1.4.1106). *P*-values for multiple comparisons were adjusted by using Tukey post hoc test for multiple comparisons. Packages exploited were *"glmmTMB"* (Brooks et al., 2017), *"plyr"* (Wickam, 2011), *"ggplot2"* (Wickham, 2016), *"MuMIn"* (Barton, 2022), and *"multcmp"* (Hothorn et al., 2008).

For the HRMS data, we used MS-DIAL software version 4.9 for the peak picking, deconvolution, noise level setting, and identification of metabolites. The identified peaks were aligned on a QC (Quality Control) sample, to also allow the monitoring

of the response of the instrument. Deconvoluted chromatograms were normalized on the Total Ion Current (TIC) and analysed through a Principal Component Analysis (PCA) to account for the effect of the sampling method followed by PERMANOVA to account for statistical significance (α = 5%) in R by exploiting the "*vegan*" package. Significant metabolites responsible for the clusterization among the experimental groups were more deeply characterized by Data Dependent Acquisition (DDA) setting as ion intensity threshold a value of 5×10^4.

Results

Pollen nutrient composition

The estimation of the nutrient composition of pollen is shown in Fig. 3. The total protein content was significantly lower in dry anthers (13.27 ± 3.80 % *w/w*) compared with the E-PoSa (15.92 ± 3.63 % *w/w*) and mesh (17.77 ± 3.82 % *w/w*). No significant differences emerged in the comparison between the total protein content in pollen collected with E-PoSa and the mesh. The lipid content was significantly lower in anthers (3.15 ± 0.94 % *w/w*) than E-PoSa (6.63 ± 0.64 % *w/w*) while in the mesh samples (4.87 ± 0.80 % *w/w*) it was comparable both to the E-PoSa and the anthers. Concerning the sugar composition of pollen, their content was higher in anthers, especially glucose (3.48 ± 1.16 % *w/w*), which showed significant differences both with E-PoSa (2.1 ± 0.79 % *w/w*) and the mesh control

group (1.24 ± 0.43 % *w/w*); similarly fructose content was higher in anthers (2.86 ± 0.65 % *w/w*) compared to the mesh (1.36 ± 0.72 % *w/w*), while the E-PoSa (1.86 ± 0.45 % *w/w*) did not significantly differ with anthers and mesh. Conversely, no significant differences among the three sampling methods were found concerning the sucrose, total phenol, total flavonoid content, and total antioxidant activity.

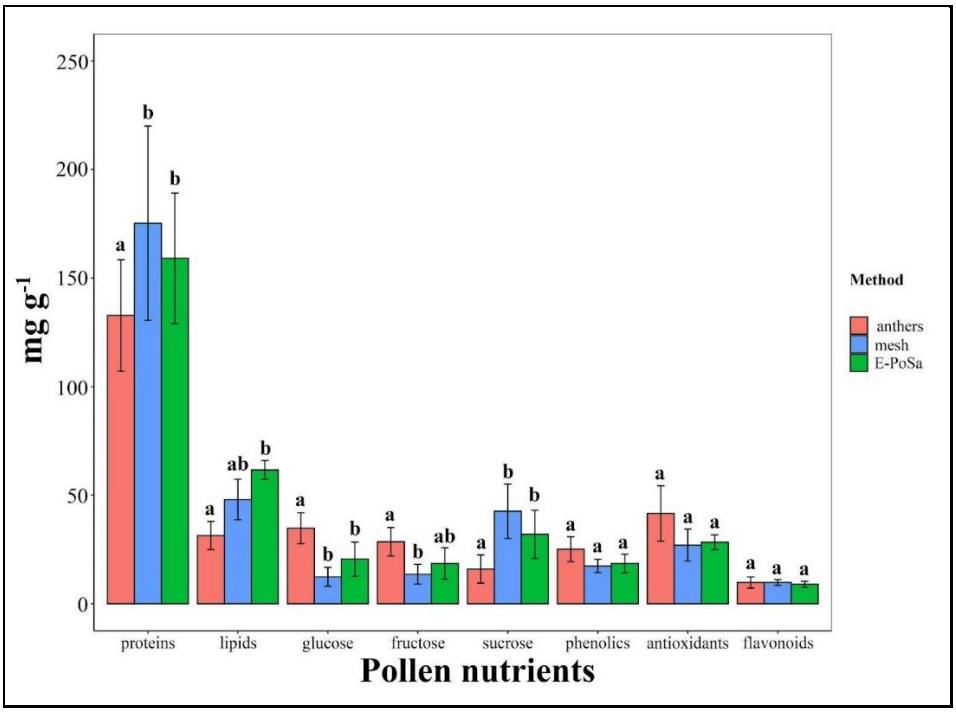

Figure 2. Quantified percentages of the nutrient composition of samples in the 3 species studied which are implemented into the regression model as a random factor. The results are shown as the mean values obtained for the three sampled species. Values are reported as the mean ± SEM (standard error of the mean). For each of the detected analytes, significant differences ($p < 0.05$) estimated through the post hoc Tukey test are declared within the same nutritional category and are reported by different letters, while in the case of non-significantly different comparisons the letters above the bar plots are the same.

Pollen phytochemicals composition

The results obtained from the HRMS analyses of the hydro-alcoholic extracts are reported in Fig. 4. Results showed that on two out of the three species (i.e., *H. vittatum* and *A. aurea*) the collection of anthers led to an estimate of the phytochemical profile significantly different from what obtained by extracting mesh or E-PoSa derived pollens (Fig. 4; Table S1). In particular, the phytochemical profile of *H. vittatum* anthers showed the occurrence of many glycosylated phenolic acids (hydroxybenzoic acid, vanillic acid, sinapic acid and 3-hydroxy-3-hydroxyphenyl-propionic acid) not findable in E-PoSa and mesh sampled pollen. Anthers also showed a different set of flavonoids compared to E-PoSa and mesh samples. A similar pattern was observed in *A. aurea*, where the E-PoSa and mesh-pollen collection led to the detection of different flavonoid glycosides compared to the anthers. Generally, anthers were characterized by the occurrence of higher diversity and quantity of phytochemicals. A detailed characterization of the discriminating compounds is reported in Table S2, S3 and S4. In *T. majus*, however, no significant differences in the phytochemical profile were found based on the collection method. These results indicate that the E-PoSa pollen sampling led to a much more accurate definition of the phytochemistry of pollen compared to anthers sampling which frequently may result in impaired metabolic profiles.

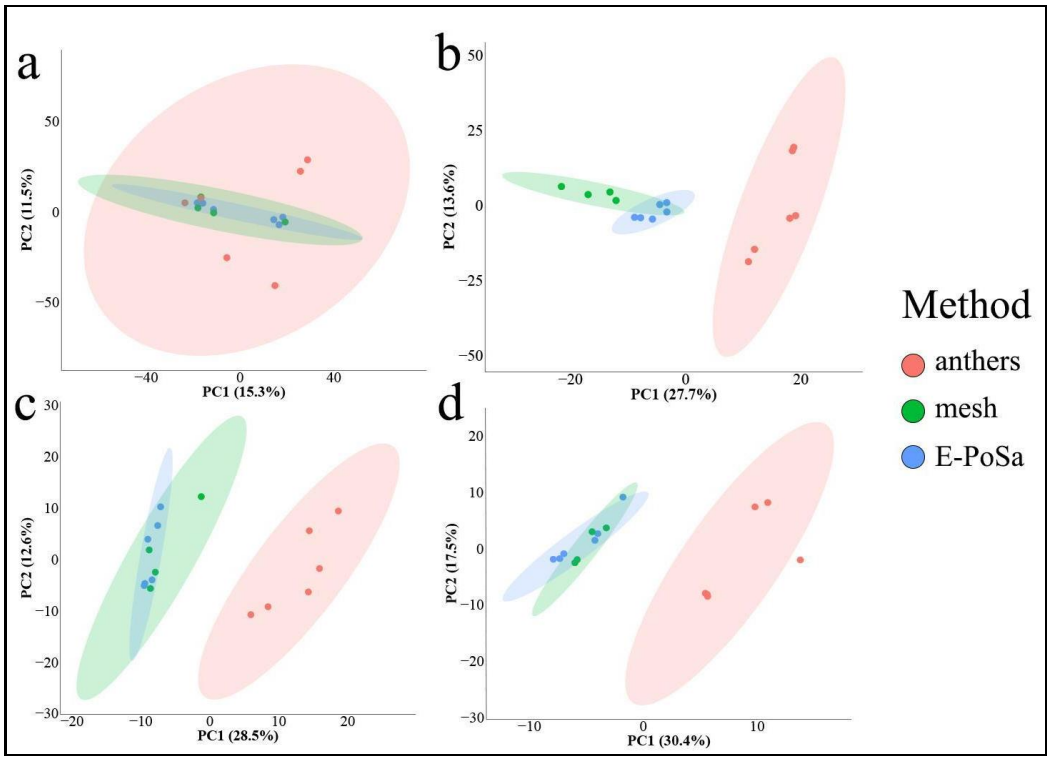

Figure 3. output of the PCA analyses performed on the HRMS chromatographic traces of hydroalcoholic extracts for *T. majus*, negative ionizing mode (a), *H. vittatum*, negative and positive ionizing mode (b and c) and *A. aurea*, negative ionizing mode (d). In red are reported anthers, in green pollen sampled with E-PoSa and in blue mesh collected pollen.

Nectar sugars composition

As shown in Fig. 5, the volume of nectar recovered varied significantly according to the method employed (*p*-value < 0.001). In detail, the volume of nectar retrieved per flower was higher by using glass microcapillaries (7.9 ± 0.54 µL per flower) or micro-rinsing (10 ± 1.22 µL per flower) with similar recovery for these two methodologies (*p*-value = 0.17), while a significantly lower amount of nectar (2.1 ±

0.08 µL per flower) was retrieved by centrifugation compared both to microcapillaries and micro-rinsing (*p*-value < 0.001). It is not possible to estimate the amount of nectar recovered by the wash method because the nectar is directly suspended in a fixed volume of water.

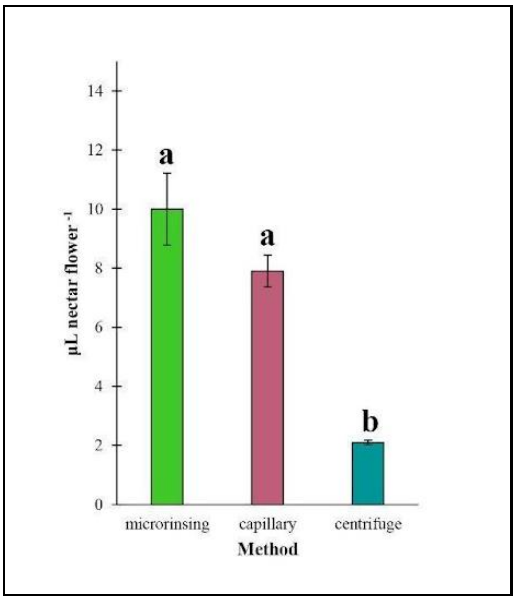

Figure 5. Barplot reporting the recovery of nectar as volume per flower for the different methodologies. The results are shown as the mean values obtained for the three sampled species. Values are reported as the mean ± SEM (standard error of the mean). Significant differences ($p < 0.05$) estimated through the post hoc Tukey test are reported by different letters.

Concerning the analysis of nectar samples, the main sugar driving the nectar composition in the three studied species was sucrose, followed by fructose and

glucose (Fig. 6). Both sucrose and total sugars content were significantly lower in micro-rinsed samples and wash compared with the centrifuge, while the microcapillary did not show significant differences compared with the other sampling strategies. Significantly higher glucose contents were found in samples collected by the wash method compared both with micro-rinsing and glass microcapillary, while the fructose content was lower in micro-rinsed samples compared both with the centrifuge and wash. Overall, compared to the glass microcapillary sampling method, the centrifugation led to the recovery of a higher amount of nectary sugars, while the other methods showed some underestimation depending on the sugar type analysed.

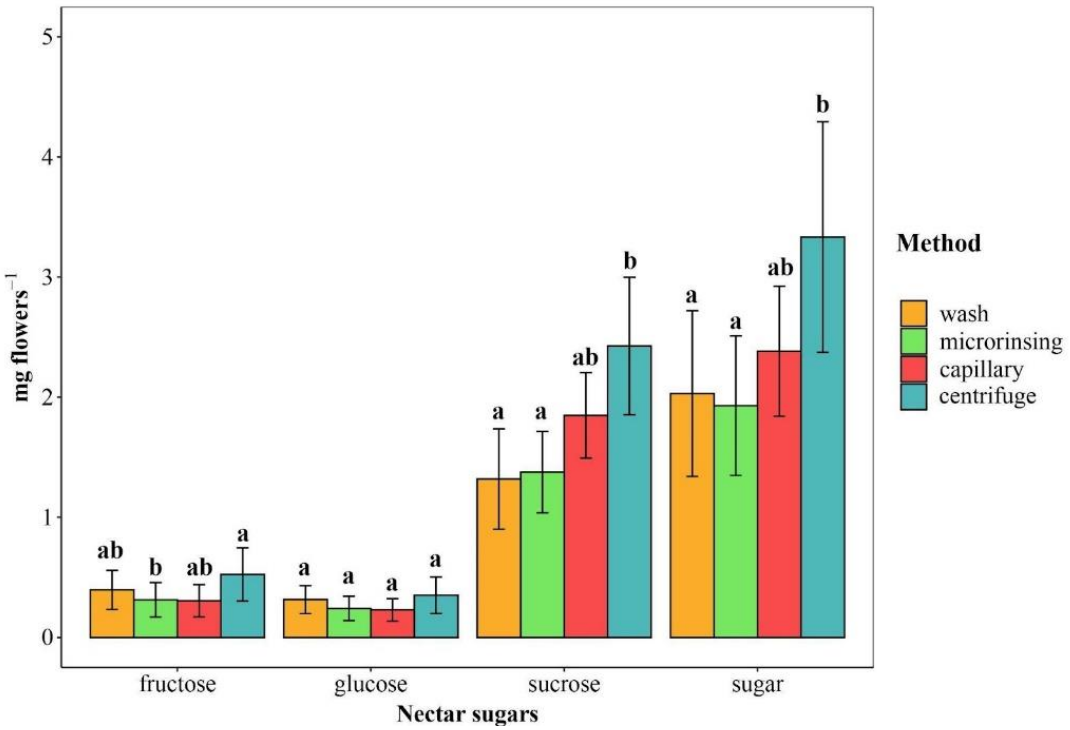

Figure 6. Quantity (mg) of sugars recovered per flower in the three studied species (see Par 2.1) based on the four studied sampling methods. The results are shown as the mean values obtained for the three sampled species. Data are reported as mean ± SEM (standard error of the mean). For each of the detected analytes, significant differences ($p < 0.05$) estimated through the post hoc Tukey test are declared in the text as well as reported by different letters, while in the case of non-significantly different comparisons letters above the bar plots are the same.

Nectar phytochemical composition

As shown in Fig. 7 and Table S6, in two out of the three species analysed (i.e., *S. greggii* and *A. praecox),* the ordination analyses performed on nectar metabolomic data showed that the microcapillary and flower centrifugation are the most

indicated methods to avoid sample contamination for nectar collection. The microcapillary sampled nectar from *R. equisetiformis* displayed higher phytochemical similarity with the one obtained through micro-rinsing than centrifugation. Generally, sampling nectar by washing resulted in phytochemical profiles contaminated by the occurrence of typically pollen-originating compounds (see Table S6, S7 and S8 for their identification), responsible for consistent biases from the actual phytochemical composition of the nectar, while methods such as centrifuge and micro-rinsing appeared to be more accurate even though not always perfectly overlapping with the results obtained by the sampling through microcapillary tubes.

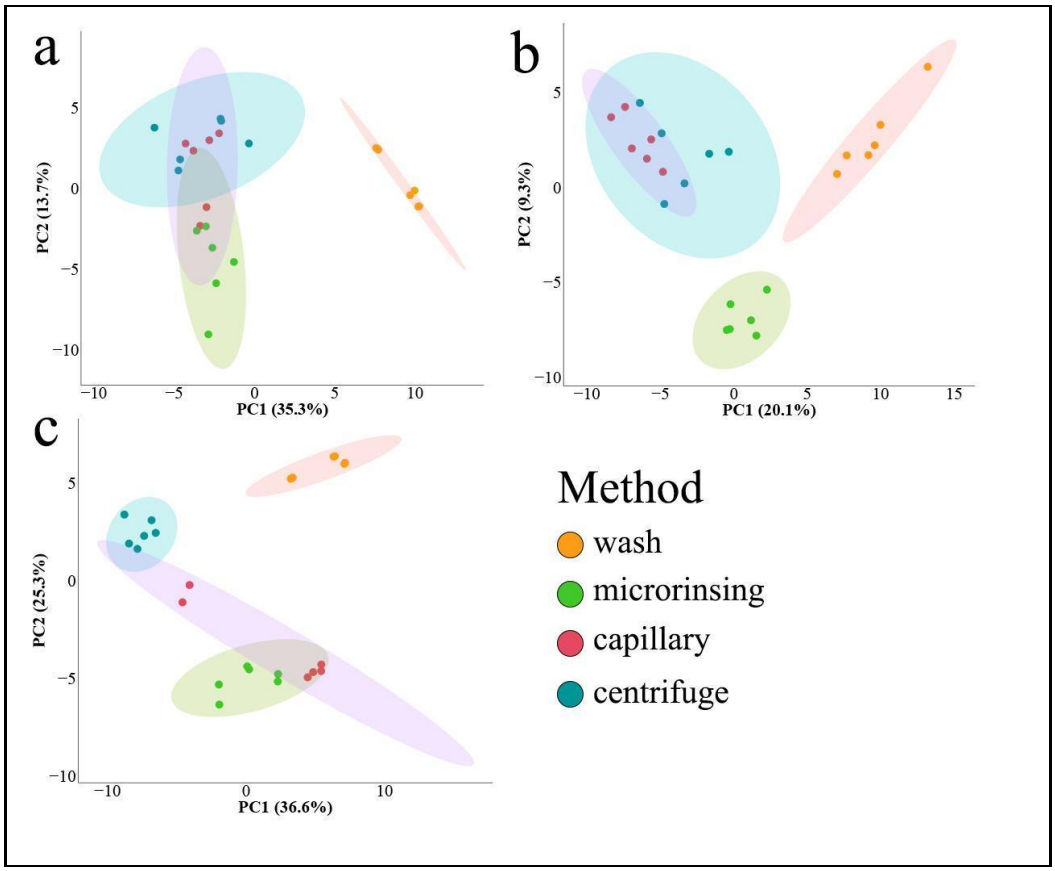

Figure 7. output of the PCA analyses performed on the HRMS chromatographic traces of nectars sampled from *S. greggii* (a), *A. praecox* (b) and *R. equisetiformis* (c).

Discussion

The recent advances in pollination nutritional ecology require the definition of standardized sampling methods for flower rewards able to overcome biases in the characterization and quantification of compounds of nutritional interest, both from an analytical and sampling point of view (Jeannerod et al., 2022; Power et al., 2018).

In this study, it is shown that the sampling technique provides for important differences in the amount of the recovered pollen and nectar and the nutritional profiling both at the macronutrients and secondary metabolites levels.

Pollen sampling and biases

The analysis of pollen from wild plants needs to be fine-tuned for an accurate definition of its role in the nutritional balance of pollinators' diets (Lau et al., 2022). It is well known that the recovery of pollen grains from wild plants is a difficult task to perform since the anthers of enthomogamous plant species usually produce low amounts of pollen (e.g., Jeannerod et al., 2022; Palmer-Young et al., 2019). However, obtaining a significant amount of pollen with no contaminants originating from other floral parts may be very time-consuming, thus reducing the efficiency and the feasibility of nutritional ecology studies. Results from the nutritional analyses of pollen performed on the three target species show that pollen collected by the E-PoSa did not show any significant difference in terms of total nutrient composition compared to the mesh sampled pollen. The exploitation of anthers bearing pollen to characterize the nutrient composition of pollen grains may result in some biases in the total nutrients profiling of samples as shown in Fig. 5. These contaminations of ectopic plant portions are even more evident when HRMS analyses are performed. For instance, we found out that the chemical composition of anthers samples is clearly biased compared to the other experimental groups,

for instance in *H. vittatum* and *A. aurea* a high content of phenolic acids not occurring in pollen grains was detected. This may result in an improper evaluation of pollinators' nutrition. The bias comes out from the flawed selection of the starting matrix and not from the effective production of toxic or health-promoting compounds in pollen grains themselves. Pollen grains sampled with the E-PoSa displayed a chemical profile like the mesh sampled ones, indicating a generally accurate sampling of the matrix of interest. However, the method resulted in a significantly higher recovery of pollen per floral unit, thus suggesting being a low time-consuming and high-yielding sampling procedure for floral pollen (Pioltelli et al., 2023).

Nectar sampling and relative biases

Nectar sampling suffers from relevant biases in the definition of the amino-acid profile if the sampling is performed with no care to avoid contamination from other floral parts such as pollen and, to a lower extent, petals (Power et al., 2018). In this study, we found that also the sugar composition of nectars is affected by the sampling technique, and this is shown regardless of different species, displaying various flower morphologies (i.e., tubular corolla in the case of *R. equisetiformis*, zygomorphic symmetry for *S. greggii* PQ variety and radial shape of *A. praecox*). The higher amount of recovered sugars per flower is found by sampling nectar by centrifugation, a method yet proposed in some studies (e.g., Russel & McFrederick,

2022; Kim et al., 2021); however, the amount of recovered total sugar is similar if nectar recovery is performed by the glass microcapillary which is among the most adopted methods for nectar extraction (Power et al., 2018). Conversely, the recovery of nectar was found to be less effective by centrifugation thus requiring a higher sampling effort compared with the other methods. Sampling flower nectar with methods providing the addition of water, such as micro-rinsing and washing, resulted in lower total sugar quantification, probably due to an incomplete recovery of the whole volume of water added. This pattern is confirmed when analysing the major sugar occurring in nectar (i.e., sucrose).

Different outcomes were found by the quantification of monomeric sugars (i.e., fructose and glucose). Despite their presence, the relative abundance is higher in the wash group, indicating that possible contaminations of other flower parts may impair the relative concentration of these compounds. However, the clearest indication of putative pollen contaminants in nectar due to the sampling method comes from three aspects. Firstly, the analysis of the secondary compounds showed that the wash method strongly separated in the ordination multivariate space from centrifugation, glass microcapillaries, and micro-rinsing. Secondly, the micro-rinsing and wash method revealed the presence of rutin, a well-known secondary compound strongly associated with the presence of pollen (Rocchetti et al., 2019). Thirdly, many other compounds belonging to the classes of flavonoids,

triterpenoids and polyamines are found exclusively or in significantly higher concentration in wash nectar samples suggesting an evident extra-nectar origin. These observations are relevant if considering that in recent years several studies have clarified the role of secondary metabolites in the definition of the nutritional value of flower rewards (Palmer-Young et al., 2019; Stevenson et al., 2017). Rutin, for instance, has been proven to act as a protective agent against the negative impacts of common insecticides (Riveros & Gronenberg, 2022). Therefore, the identification of such kinds of compounds when not occurring within nectar or their over-quantification may result in a great bias for the definition of the nutritional features and value of flower rewards. Furthermore, the biases in the estimation of the sugar content of nectar could produce misleading interpretations in research dealing with plant-pollinator interactions as multiple studies observed species-specific preferences for nectar sugars (Woodcock et al., 2014; Kelber, 2003; Romeis & Wackers, 2000).

Conclusions

The field of nutritional ecology in the context of plant-pollinator interactions is growing in importance, to address the issues of pollinators safeguarding and conservation. The comparison among the many sampling techniques performed in the present study strengthens the need for a standardization of the methodological

tools adopted by the scientific community to better compare the nutritional outputs of studies dealing with the evaluation of flower rewards. The evaluation of the reliability of novel sampling techniques compared to the traditional ones shed new light on how to perform effective and affordable sampling of floral resources to address nutritional ecology-oriented studies.

ACKNOWLEDGEMENTS

Funder: Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU.

Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP, H43C22000530001 Project title “National Biodiversity Future Center - NBFC”. The authors are grateful to Barbara Ruffoni, Paolo Mussano, and the team of the CREA of Sanremo for their support. A special thank goes to Fausto Ramazzotti and Luca Tonietti for their help during the sampling activity. The author thanks Maura Brioschi for her support with the mass spectrometry analysis.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest.

AUTHOR CONTRIBUTIONS

EP, LG, WGN conceived the ideas and designed the methodology; EP, LG and AC collected the data; EP and LG analyzed the data; EP, LG, AG, WGN and PB led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Appendix S1

Text S1: E-PoSa assembly and use

E-PoSa is design utilizing a portable vacuum cleaner with a removable plastic mask. Its components include a portable vacuum cleaner, a 5 mL tube with a trimmed head and the lid drilled, an inox mesh sheet, a paper filter, and laboratory film (Figure S1). The first step for assembling the device is preparing the 5 mL tube by cutting its head approximately 0.5 - 1 cm from the tip using a sharp knife. Secondly, the tube lid must be separated and drilled by using a conical drill bit. The next step is cutting the paper filter with a diameter slightly larger than that of the test tube cap. Then, close the tube by paying attention that the paper filter remains in the correct position by completely covering the hole previously made on the cap. A small square of the inox mesh sheet is heated briefly using a lighter, placed on top of the tube, and held in position for a few seconds to ensure it does not detach. The final step is to connect the tube to the vacuum head and secure it using the laboratory film. The entire assembly process is estimated to take less than 10 minutes. A few seconds are required for re-placing the pre-assembled collection tubes. The battery life of the portable vacuum used in this study is about 30 minutes.

The use of E-PoSa does not require expertise. Pollen collection is achieved by activating the vacuum and gently moving it over the target flowers. Pollen is

aspirated and accumulates on the paper filter within the tube. Due to the transparency of the tube, the amount of pollen collected can be easily estimated by eye and once enough pollen is collected, it can be directly transferred to another tube by removing the inox mesh from the tip of the E-PoSa and gently tapping on the bottom of the adapted tube. The system is adaptable to flowers of different morphologies. For smaller flowers or those with concealed anthers within the corolla (e.g., Lamiaceae or Fabaceae) a 20 µL tip can be attached to the tube and fixed with laboratory film enabling more precise and effective pollen sampling.

Fig. S1. Assemblage of the E-PoSa. 1. Overview of all the materials needed for the assembly of the E-PoSa device (a. Portable vacuum; b. Paper filter with 25 µm pore size; c. 5 mL Eppendorf tube's cap drilled; d. 5 mL Eppendorf tube with Stainless steel mesh with 75 µm mesh size at the tip; e. strip of laboratory film); 2. Top view of the fully assembled E-PoSa 3. Frontal view of the fully assembled E-PoSa.

Text S2: HPLC-ESI-qToF-MS/MS analysis

Samples were randomized and used for HRMS analyses by using a Waters ACQUITY UPLC system coupled with a Waters Xevo G2-XS QTof Mass Spectrometer (Waters Corp., Milford, MA, USA). All analytes were separated on a UPLC system equipped with a Zorbax SB-C18 column (100 mm × 2.1 mm, 3.5 µm). The mobile phases were both MS grade H_2O (A) and MeCN (B), both containing 0.1% formic acid (HCOOH), with gradient elution from 5% B to 95% B in 25 minutes. After each run, the column was washed for 5 mins (95% B) and then equilibrated for further 5 mins at the initial conditions (5% B) before the next sample injection. For nectar samples, due to the high concentration of free sugars, the gradient was maintained isocratically at 5% B for 3 minutes (without reaching the source) before starting with samples elution and acquisition. Elution was performed at a flow rate of 0.4 mL/min, and the injection volume was 10 µL for pollen samples and 5 µL for nectars. The concentration of the injected samples was equal to 0.1 mg/mL for pollen extracts and nearby 1 mg/mL for nectars. The column temperature was set at 30°C. The Xevo G2-XS QTof Mass Spectrometer, equipped with an ESI source, was used both in positive and negative ionization mode to acquire full-scan MS and the spectra were recorded in the range of m/z 100–1200 Da. The source parameters were as follows: electrospray capillary voltage 2.0 kV, source temperature 140°C, and desolvation temperature 600°C. The cone and desolvation gas flows were 0 and 1000 L/h,

respectively. A scan time of 0.5 s was employed. The mass spectrometer was calibrated with 0.5 M sodium formate and leucine-enkephalin (100 pg/µl) was used as LockMass (m/z 554.2615, 0.8 kV ionization voltage for negative mode; m/z 556.2677, 0.8 kV ionization voltage for positive mode), which was infused simultaneously with the flow of column at 8 µl/min and acquired for 1 s each 30 s. The MassLynx software 4.2 was used for instrument control, data acquisition, and data processing. To obtain information about the identity of full-MS detected compounds, the chromatographic runs of some representative samples were carried out in Data Dependent Acquisition mode, with a range for fragment identification between 50 and 1200 Da. The collision energy applied ranged from 20 to 30 V for compounds exhibiting low masses and was between 30 and 50 V for high molecular masses. The scan time was 0.1 second and the threshold was each time varied according to the relative intensity of the lowest signals of compounds occurring in the full scan traces. As a preliminary step of the analysis, for each species, we injected a QC sample to see the occurrence of compounds and the ionization mode characterizing them. For all the species analysed, the negative ion current runs were characterized by complex chromatographic profiles while the positive current runs were not informative (scarce compounds and/or most of them already found in the negative mode). The only exception was made for *H. vittatum*,

whose chromatographic profile was found to be informative also in the positive ion mode.

Table S1: Pollen metabolomics statistical results

Tropaleum majus (neg)	**anthers**	**E-PoSa**
E-PoSa	0.084	NA
mesh	0.522	0.309

Hippeastrum vittatum (neg)	**anthers**	**E-PoSa**
E-PoSa	< 0.01*	NA
mesh	0.015*	0.279

Hippeastrum vittatum (pos)	**anthers**	**E-PoSa**
E-PoSa	< 0.01*	NA
mesh	0.018*	0.162

Alstroemeria aurea (neg)	**anthers**	**E-PoSa**
E-PoSa	0.018*	NA
mesh	0.024*	0.078

Table S2: MSMS identification of typical anthers occurring compounds in Hippeastrum vittatum in negative scan.

Rt	m/z	MSMS fragments	%B	ID	Reference	anthers vs others ($p < 0.05$)
1,65	299	137, 93, 218, 280	8	Hydroxybenzoic acid hexoside	Kramberger et al., 2020	↑
2,36	329	167, 152, 123, 108	11	Vanillic acid hexoside	Zengin et al., 2019	↑
2,64	343	181, 163, 135, 119	12	3-hydroxy-3-hydroxyphenil-propionic acid	Zhu et al., 2022	↑
4,35	369	223, 205, 111, 101	18	Sinapic acid rhamnsoside	Ferreres et al, 2006	↑
4,5	399	223, 205, 129, 111, 101	20	Sinapic acid glucuronide	Chang et al. 2016	↑
5,21	755	593, 447, 285	22	Luteolin dihexose desoxyhexoside	Sahin et al., 2013	↑
6,15	475	343	27	di-hydroxy caffeic acid hexoside pentoside	Huang et al., 2015	↑
6,4	593	285, 255	28	Kaempferol-3-O-rutinoside	Huang et al., 2015	↓
7,17	287	177, 151, 125,	30	7,3'-4'-5'-Tetrahydroxy B	Chen et al., 2014.	↑

		107		flavanone		

Table S3: MSMS identification of typical anthers occurring compounds in Hippeastrum vittatum in positive scan.

Rt	m/z	MSMS fragments	%B	ID	Reference	anthers vs others ($p < 0.05$)
6,65	287	258, 213, 153, 121	28	Kaempferol	MS DIAL	↓

Table S4: MSMS identification of typical anthers occurring compounds in Alstroemeria aurea in negative scan.

Rt	m/z	MSMS fragments	%B	ID	Reference	anthers vs others ($p < 0.05$)
2.7	445	137, 93	12	Hydroxybenzoic acid hexoside rhamnoside	Kramberger et al., 2020	↑
4.04	293	131	17	uknown hexoside		↑
4,98	755	593, 284	21	Kaempferol/Luteolin-o-hex-DHex-o-Hex	Public library (MS DIAL)	↑
5,75	593	447, 446, 301, 300, 298, 271	25	Quercetin-di-desoxyhexoside	Public library (MS DIAL)	↑
5,85	563	430, 283	25	Resokaempferol di-O-hexoside	Public library(MS DIAL)	↑
6.12	623	315, 314, 300	27	Isorhamnetin-o-rutinoside	arros et al., 201	↓
6,21	577	255, 284, 285, 300, 431	27	Kaempferol 3,7-di-O-rhamnoside	Barros et al., 2011	↑

6,39	573	285, 284, 255, 227	27	Kaempferol derivative	Li et al., 2016	↑
6,54	771	609, 463, 301	28	Quercetin-7-O-glycoside-3-O-rutinoside	Brito et al., 2014	↓

Table S5: Nectar metabolomics statistical results

Salvia greggii (neg)	**capillary**	**centrifuge**	**rinsing**
centrifuge	0.522	NA	NA
rinsing	0.096	< 0.01*	NA
wash	0.03*	0.024*	0.012*

Agapanthus praecox (neg)	**capillary**	**centrifuge**	**rinsing**
centrifuge	0.078	NA	NA
rinsing	0.018*	0.036*	NA
wash	< 0.01*	< 0.01*	0.036*

Russelia equisetiformis (neg)	**capillary**	**centrifuge**	**rinsing**

centrifuge	< 0.01*	NA	NA
rinsing	0.432	0.036*	NA
wash	0.024*	0.012*	0.036*

Table S6: MSMS identification of typical washing occurring compounds in Salvia greggii in negative scan.

Rt	m/z	MSMS fragments	%B	ID	Reference
5,06	355, 0706	309, 292, **209**, **191**, 129, 85	10	Coumaroyl glucarate isomer	Csepregi et al., 2020
7,8	387,1665	371, 309, 207, 163, 101, 89	15	Medioresinol	Ozarowski et al., 2013
9,39	595,1332	301, 300, 271, 255, 179, 151	20	Quercetin-hexoside-pentoside	Llorent-Martínez et al., 2017
9,92	609,1491	301, 300, 271	21	Rutin	Public library (MS DIAL)
10,23	463,0908	351, 325, 302, 301, 285, 284, 255, 151	22	Quercitin-3 -O galactodise	Public library (MS DIAL)
10,65	579,1739	295, 271, 151, 119	23	Naringenin -7-O-rutinoside	Public library (MS DIAL)
11,08	447,0965	284, 285, 255, 227	24	Kaempferol-3-O-glucoside	Carvalho et al., 2022
11,28	609,1895	343, 325, 301, 242	24	Hesperidin	Public library (MS DIAL)

13,31	593,1912	327, 309, **286**, **285**	27	Isosakuranetina -7-O-rutinoside	Public library (MS DIAL)
17,1	345,1725	284, 283, 268, 227	55	Rosmanol	Pedro Mena et al., 2016
17,46	311,1303	283, 268, 255, 239, 183	55	Calychosin derivative	Ye et al., 2012

Table S7: MSMS identification of typical washing occurring compounds in Agapanthus praecox in negative scan.

Rt	m/z	MSMS fragments	%B	ID	ReferenceReference
6,68	817.2	655, 493, 331, 316	12	Laricitrin 3,5,7-triglycoside	Public library (Compound Discoverer)
8,07	609.1	301	15	Quercetin-3-O-rutinoside	Public library (Compound Discoverer)
9,29	579.1	447, 315, 300, 271	19	Carlinoside isomer	Public library (Compound Discoverer)
10.5	1159,5	1113, 951, 935, 917, 789, 735, 723, 627, 609, 247, 179	25	unknown tri-O-hexoside (formate)	
11.6	1143.7	1097, 967, 951, 787, 739, 643, 625, 307, 247, 179, 163	30	unknown rhamnosyl-O-dihexoside (formate)	

Table S8: MSMS identification of typical washing occurring compounds in Russelia equisetiformis in negative scan.

Rt	m/z	MSMS fragments	%B	ID	Reference
5,95	813	285, 351, 527	21	Kaempferol-O-triglucuronide	Maietta et al., 2018
6,5	797	113, 193, 269, 351, 527	22	Apigenin-O-triglucuronide	Maietta et al., 2018
6,65	621	113, 193, 269, 289, 311, 351	23	4-O GlcA (1,2) GlcA Apigenina	Public library (MS DIAL)
6,9	523	135, 161, 179	25	Verminoside	Friščić et al., 2016
7,68	507	119, 145, 163, 345	27	Specioside	Friščić et al., 2016
7,94	537	160, 175, 193, 261, 375	30	Minecoside	Friščić et al., 2016
8,1	507	119, 145, 163, 345	30	Specioside	Friščić et al., 2016
9,54	582	119, 145, 299, 316, 342, 462	40	Tricoumaroyl-spermidine isomer	Public library (MS DIAL)

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Case study III. Pioltelli, E., Guzzetti, L., Ouled Larbi M., Celano R., Piccinelli A.., Galimberti A., Biella P. & Labra M. (2023). Land use influences the nutrient concentration and composition of pollen and nectar rewards of wildflowers in human-dominated landscapes.

Type of article: Research paper

Status: Accepted in “Science of the total environment”

Land use influences the nutrient concentration and composition of pollen and nectar rewards of wildflowers in human-dominated landscapes.

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Keywords

Nutritional ecology - Pollen and Nectar - Pollinators diet - Landscape anthropization – Plant metabolism

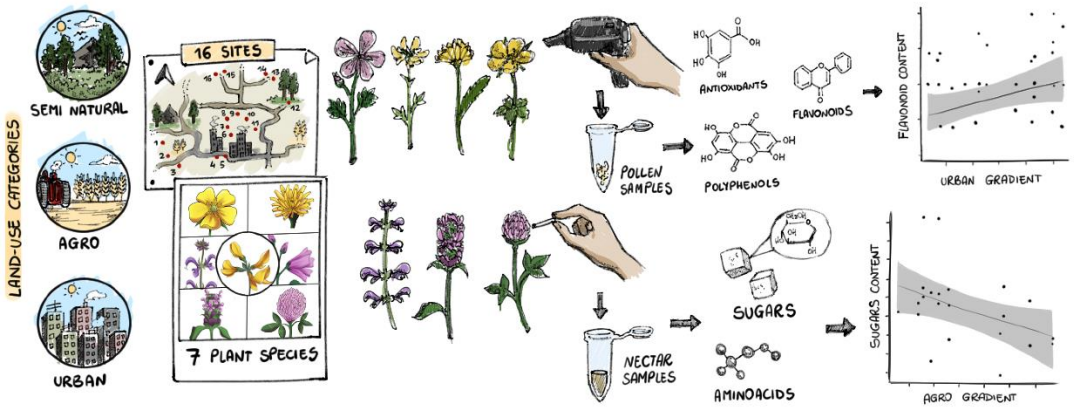
Data availability

Data are available at https://doi.org/10.6084/m9.figshare.24026328.v1

Highlights

- Few studies have explored the impact of land use on floral reward chemistry and nutritional value.
- 7 meadow species were studied for nutritional traits across both urbanization and agriculture gradients.
- Both plant primary and secondary metabolism were investigated by means of analytical chemistry.
- Both nectar sugar content and pollen antioxidant activity were influenced by land use.
- Results offer novel insights for restoration ecology strategies in human-modified environments

Graphical abstract



Abstract

Plant biodiversity is crucial to satisfy the trophic needs of pollinators mainly through nectar and pollen rewards. However, a few studies have been directed to ascertain the intraspecific variation of chemical features and the nutritional value of floral reward nectar and pollen in relation to the alteration of landscapes due to human activities. In this study, by using an existing scenario of land use gradients as an open-air laboratory, we tested the variation of pollen and nectar nutrient profiles along gradients of urbanisation and agriculture intensity, by focusing on sugar, amino acids of nectar and phytochemicals of pollen from local wild plants. We also highlighted bioactive compounds from plants primary and secondary metabolism due to their importance for insect wellbeing and pollinator health. We surveyed 7 different meadow species foraged by pollinators and common in the main land uses

studied. The results indicated that significant variations of nutritional components occur in relation to different land uses, and specifically that the agricultural intensification decreases the sugars and increases the antioxidant content of flower rewards, while the urbanization is positively associated with the total flavonoid content in pollen. These effects are more evident in some species than in others, such as *Lotus corniculatus* L. (Fabaceae) and *Malva sylvestris* L. (Malvaceae) as shown by the untargeted metabolomic investigation. This study is crucial for understanding the nutritional landscape quality for pollinators in association to different land uses and sets a base for landscape management and planning of pollinator friendly strategies by improving the quality of plant reward for providing benefits to pollinator health in various environmental contexts.

Introduction

The progressive expansion of urban and agricultural areas is associated with a series of environmental modifications that greatly impact urban and periurban plants and pollinators biology (Biella et al., 2022; Tommasi et al., 2022). Since many pollinators base their nutrition on flower rewards, which have an impact on their development and population size, changes in the environment, that also modifies nectar and pollen, could also affect the conservation of pollinators and the pollination ecosystem services at several levels. Therefore, studying the intraspecific variation of flower resources composition is urgent

(Venjakob et al., 2020), especially in relation to human-induced environmental alterations to understand the magnitude of the ongoing impact and so to supply landscapes or mitigate situations of lack of nutrients for pollinators. This is particularly relevant within the process of ecological transition and intensification towards sustainable cities and agricultural areas, and to plan actions capable of guaranteeing an adequate quality of floral resources for pollinators (Jones & Rader, 2022).

Flowering plants and pollinators are entangled by reproduction and foraging needs, respectively. Specifically, flower visitors mainly depend on pollen and nectar to satisfy their nutritional requirements (Nicolson et al., 2018). For instance, nectar is the main food for the adult forms of various pollinators and pollen is the main food for the larval stages of bees or some adult hoverflies and beetles (Faegri & Van Der Pijl, 1979). These resources display relevant differences in their chemical and nutritional composition, mainly in relative amounts of the main components. Nectar is a solution of mainly sugars that supports the essential needs of the energetic metabolism of pollinators, but it also contains several compounds, such as amino acids, and phytochemicals (Barberis et al., 2021; Roy et al., 2017). These latter compounds are relevant to multiple aspects of pollinators ecology as they can manifest as either attractive agents or deterrents, thereby exhibiting a substantial range of effects that are frequently contingent upon dosage (Stevenson et al., 2017; Manson et al., 2013). Pollen chemistry is mainly composed of lipids, proteins, amino acids, and it is characterized by a high diversity and concentration of secondary metabolites (Palmer Young et al., 2019; Thakur, M. & Nanda, 2020; Aylanc et al., 2021).

Pollinator diet on flower resources is an essential factor for them to counteract the negative impact of anthropogenic stressors through the provision of essential nutrients from plant primary and secondary metabolism (Barascou et al., 2021; Wong et al., 2018). To guarantee an adequate dietary intake in space and time for the different species of pollinators, it is essential to understand which are the environmental parameters that influence macro and micronutrients composition of pollen and nectar. Many environmental variables play a role on the floral reward chemical features, especially in the quantitative variation of phytochemicals (Zu et al., 2021; Palmer Young et al., 2019). Indeed, the metabolism of plant secondary compounds is known to be highly responsive to environmental biotic and abiotic factors, among which light, temperature, and drought, as well as biotic agents, such as the damage produced by herbivores and parasites (Khare et al., 2020). However, a landscape-scale investigation of pollen and nectar quality is needed. This because most studies related to the investigation of pollinators diet quality as a function of the landscape considered the variations in the food resources directly collected by a few model species (Pioltelli et al., 2023a; Vaudo et al., 2018; Donkersley et al., 2017). From the plant side, so far most of the studies focused on landscape induced changes in the structure of the flora by means of species composition and diversity (Hou et al., 2023), or indirectly via the pollen (Biella et al 2022). However, regarding the rewards to pollinators, only a few studies focused on the modifications of the nutritional landscape represented by the plant communities in different contexts and the variation of the chemical composition of community nectar in response to environmental pressures (Tew et al., 2021; Biella et al., 2022). Moreover, a conceptual link between the availability of specific pollen species in the environment and

the functioning of bee populations was previously detailed by highlighting the importance of pollen key nutrients in the landscape (Filipiak et al., 2022). In this context, still little is known on how land use gradients modify the intraspecific variation of the chemical composition of both the main flower rewards of nectar and pollen.

The general goal of this study is to investigate the changes in the nutritional - chemical profile of some representative species foraged by pollinator insects along gradients of human-altered landscapes. Specifically, based on what outlined above and on field and laboratory evidence that abiotic features shape flower rewards (Venjakob et al., 2020, Akter & Klecka, et al 2022), we expected to observe changes in nectar and pollen of wild flowering plants in relation to varying types of land-use. To investigate this aspect, we aimed to quantify the effect of the main land use gradients associated with human presence and activities (i.e., urbanisation, agriculture intensification, temperature) on pollen and nectar from wild plants. Furthermore, the second objective was to analyse the individual nutritional components of pollen and nectars, (i.e., macronutrients and micronutrients) with reference to bioactive molecules capable of promoting the insect's well-being. This investigation is useful to better design the Nature-Based Solutions (NbS) suitable to promote functional biodiversity of future cities as claimed by the European Green Deal and the UN Agenda 2030. To address this issue, we conducted a field sampling, coupled with an array of chemical analyses to investigate the variations occurring in the flower rewards provided by some commonly visited meadows species.

Material and methods

Floral resources sampling

To study the floral resources composition of urban, peri-urban, semi-natural and rural areas, 16 sites (Fig.1) with different environmental characteristics in terms of anthropization levels were selected. The sites were specifically selected to represent highly contrasting land use conditions. Half of the sites were in areas predominantly characterized by seminatural hay meadows, while the other half exhibited a high degree of impervious surfaces (i.e., concrete, buildings, and asphalt). The selection of these sites was based on data obtained from regional land use cartography (DUSAF 6.0, available at https://www.dati.lombardia.it/Territorio/Dusaf-6-0-Uso-del-suolo-2018/7rae-fng6), and accessibility was verified prior to selection. Sites were located in the North of Italy and spread across different provinces. The sampling of pollen and nectar took place in June 2021.

The plant species target for this study are detailed in Table 1 and belong to different families and flower morphology. Species were selected to provide for a wide range of plants foraged by pollinator insects and because they were representative, in terms of availability, in the study area. Some of the selected plant species were more suitable for the sampling of nectar due to floral zygomorphic symmetry which favors

nectar accumulation in the corolla, while others displayed easier access to pollen grains, such as those radial shaped.

The randomly selected floral units of each species were covered with a nylon mesh 24 h prior to the sampling to avoid possible depletion of the resources by pollinator visits (Biella et al., 2021). Pollen was sampled by using a specifically adapted portable vacuum (E-PoSa, Pioltelli et al., 2023b) modified to have a filtrating unit consisting of a specifically perforated 5 mL tube and two filtering systems composed of stainless-steel mesh and filter paper. Nectar was sampled by using commercially available 5 µL and 10 µL glass capillary (Merck, Germany). Pollen and nectar collection was performed during the same day at each site in an hour range between 10 am and 12 am for all the species to minimise as much as possible biases due to the daytime. Samples were stored in a 1:1 v/v nectar/EtOH ratio to avoid microbial-mediated degradation of the occurring compounds (Power et al., 2018). Once in the laboratory, the nectar/EtOH solutions were dried in a tube under gaseous nitrogen and subsequently resuspended in 500 µL of ultrapure Milli-Q H_2O and stored at 80°C up to the analyses. Pollen samples were freeze-dried and stored at -80°C up to the analyses.

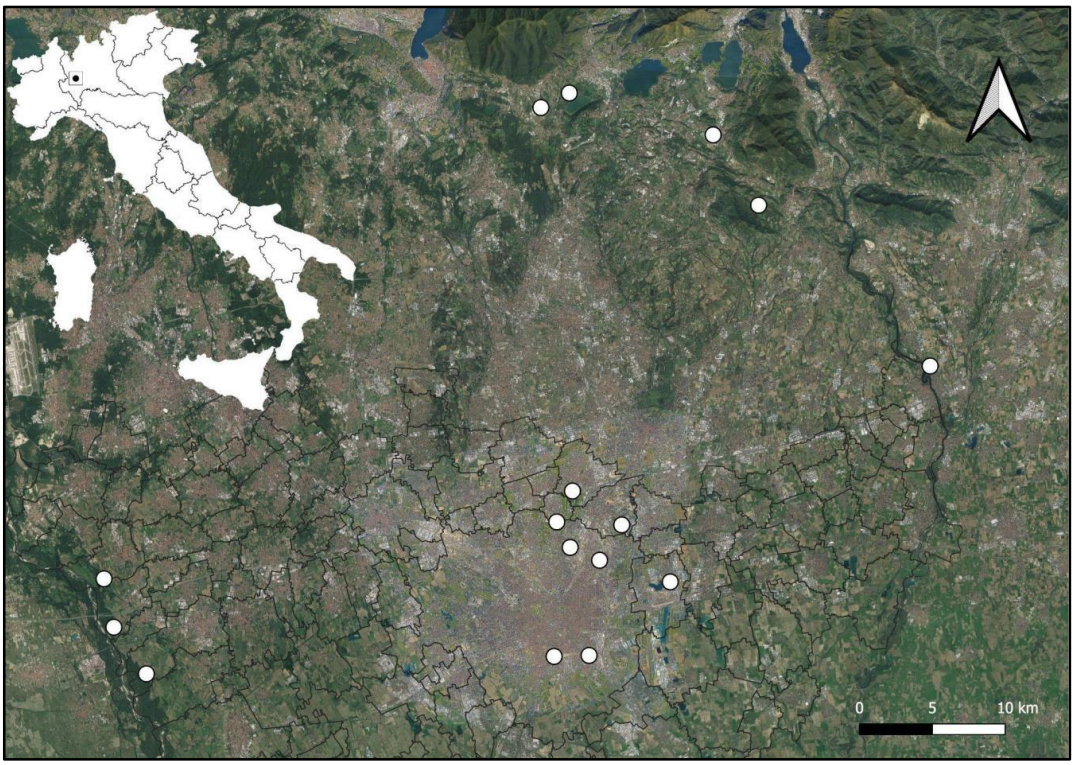


Figure 1. Map of the sampling sites where pollens and nectars analysed within the present study were collected.

Landscape and local metrics

Land cover data were obtained from the regional land use cartography (DUSAF 6.0 https://www.dati.lombardia.it/Territorio/Dusaf-6-0-Uso-del-suolo-2018/7rae-fng6). This map is available at a scale of 1:10.000 with a minimum linear dimension of polygons of 20 m and was developed from AGEA orthophotos and SPOT 6/7 satellite images. The original level and sub-level of land use classification were grouped into 3 main classes: impervious cover (i.e., concrete, asphalt, buildings),

semi-natural (i.e., hay meadows surrounded by forest), agricultural fields (i.e., agriculture margins). Detailed information on land use classification is reported in Text S1 and Table S1. Using QGIS v 3.10.11 we computed buffers of 1 km radius around each sampling location and the coverage percentage of the 3 different classes of land use were calculated.

Land surface temperature information were obtained from data retrieved through remote sensing imaging spectroradiometer (MODIS) MOD11A2 from the NASA database (https://modis.gsfc.nasa.gov/data/dataprod/mod11.php) with a resolution of 1 km. The original raster layer with a resolution of 1 km was downscaled to a finer resolution of 100 m with bilinear interpolation and the mean temperature in June 2021 was calculated starting from the mean daily registered temperature.

With the data regarding the landscape features (i.e., impervious cover, semi-natural and agricultural) and with those relative to the temperature, a Principal Component Analysis was performed in R (version 2023.06.0+421) to summarise the variability of the gradient across the different sampling sites. The principal component analysis on the landscape and climatic variables showed two main PCs complying with the two investigated land use gradients (Fig. 2). In particular, the PC1, which accounts for almost 80% of the variability of the data, was clearly defining the urbanization gradient with sites at higher impervious surface and higher temperatures associated

with high values of the PC. The PC2 which accounts for 16% of the variability in the data was associated with a gradient spanning from semi-natural areas (low values) to agricultural sites (high values).

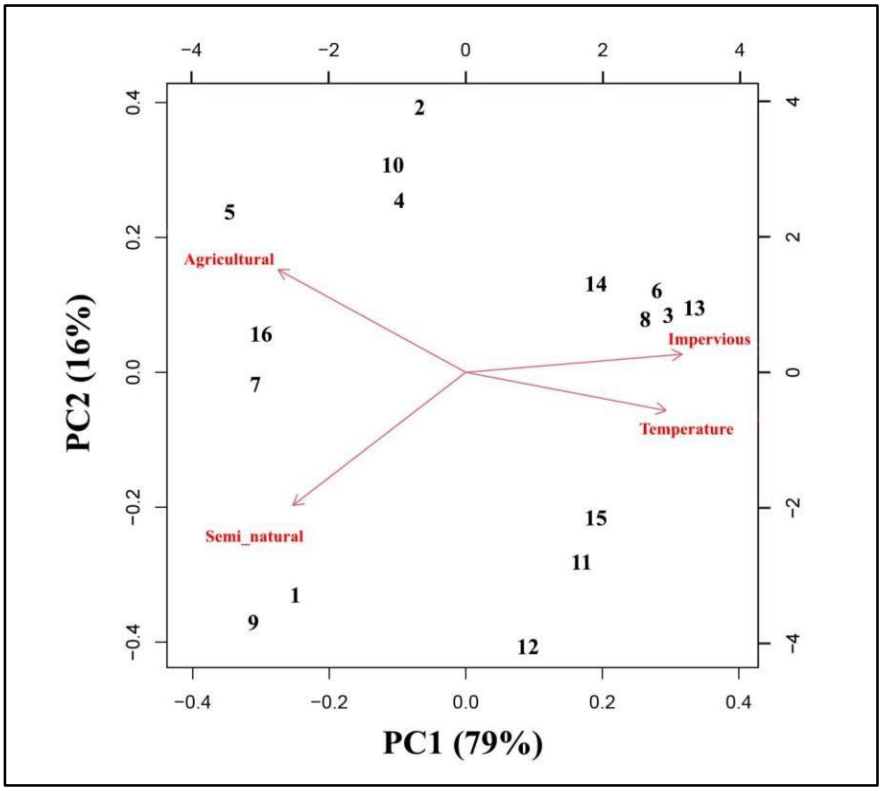


Figure 2. Principal component analysis with the landscape and environmental features of the sampling sites. The arrows indicate the direction where a particular category is higher and let the definition of two different landscape gradients.

Phytochemical analysis

Nectar and pollen were subjected to a set of analytical chemistry experiments aimed at evaluating the variations occurring in the small molecules (lower than 1200

Da) composition of flower rewards. Table 1 reports a synthetic overview of the metabolic investigations performed on nectar and pollen; further details are provided in the next paragraphs.

Table 1. Overview of the main chemical investigations performed in the present study on floral rewards, with details on their importance to the diet of pollinator insects, unit of measurement, sample size, statistical approach for their analysis, and species investigated.

Floral rewards	Species	Floral shape	Analytes	Unit of measurement	Value as a reward	*N*	Statistical analysis
Nectar	*1. Trifolium pratense* L. (Fabaceae) *2. Prunella vulgaris* L. (Lamiaceae)*3. Salvia pratensis* L. (Lamiaceae)	1. Keel/Flag flower 2. Gullet flower 3. Gullet flower	Sugars (intended as the sum of glucose, fructose, and sucrose) TSC	µg/ µL	Support to the insect energy metabolism	22	Untransformed data. Gaussian distribution
			Aminoacids (intended both as total content as well as essential aminoacid content) TAA/EAA	ng/ µL	Nutritional needs, fitness, attraction	22	Untransformed data. Gamma distribution (non normal data)
Pollen	*1. Potentilla reptans* L. (Rosaceae) *2. Lotus corniculatus* L. (Fabaceae)	1. Bowl flower 2. Keel/Flag flower 3. Ray flower 4. Bowl flower	Phenolic compounds (TPC)	µg /mg	Defence against parasites infection and pesticides detoxifiers	58	Min-max rescaled data. Beta distribution.

	3. Hypochaeris radicata L. (Asteraceae) *4. Malva sylvestris* L. (Malvaceae)		Antioxidant activity (TEAC)	µg /mg	Evaluation of the overall ability to counteract oxidative related stress phenomena	58	Min-max rescaled data. Binomial distribution.
			Flavonoids (TFC)	µg /mg	Defence against parasites infection and pesticides detoxifiers	58	Min-max rescaled data. Beta distribution.
			Untarget metabolome	NA	Identification of specific phytochemicals endowed with different bioactive properties to pollinators	58	Ordination analysis (RDA) and random forest

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Nectar sugar composition

Once resuspended, nectars were 100-fold diluted and analysed for the content of free sugars (sucrose, glucose, and fructose) by an enzymatic kit provided by Megazyme, Ireland. For the analysis of sucrose 100 µL of β-fructosidase are added to 50 µL of sample (or H_2O for the blank) followed by an incubation of 5 min. Consequently, the analysis of free sugars (glucose and fructose) starts by mixing 50 µL of sample/H_2O to 1050 µL of H_2O, while for the sucrose analysis, 950 µL H_2O is added. Then, for both analyses, we added 50 µL buffer pH 7.6 and 50 µL of a solution $NADP^+$/ATP followed by an incubation of 3 min.

At this point, the absorbance (A_1) is read at 340 nm to monitor the basal level of each sample (against the blank). Then the analysis foresees the addition of 10 µL of the enzymes hexokinase and glucose 6-phosphate dehydrogenase followed by an incubation of 5 mins. Then the absorbance is read at 340 nm (A_2). The difference value between A_2 and A_1 refers to the glucose content. Finally, 10 µL of phosphoglucose isomerase are added and each sample is incubated for 10 mins at room temperature. Then the absorbance is read again at 340 nm (A_3). The difference value between A_3 and A_1 refers to the fructose content. The content of sucrose is obtained by the difference between the absorbances of samples incubated without and with the enzyme β-fructosidase. Data on the concentration were normalised to

the volume of nectar recovered and expressed as µg/ µL nectar**.** Total sugar content (TSC) was calculated as the sum of glucose, fructose, and sucrose.

Nectar amino acids content

The nectar amino acids composition was evaluated by UHPLC (Ultimate Dionex 3000, ThermoFisher, USA) coupled with a UV and fluorescence detector. The analytes were separated with a Kinetex C-18 column (2.1 x 100 mm, 2.6 µm) coupled to a column guard (Phenomenex, USA). The mobile phases were (A) NH_4COOH 5 mmol/L, pH 7.8 and (B) MeCN/MeOH/H_2O 45:45:10 v/v/v. The chromatographic gradient was made as follows: 0-0.5 min 2% B, 2 min 15% B, 3 min 25% B, 4 min 35% B, 5 min 45% B, 10 min 70% B, 11- 15 min 98% B. The column was conditioned at 2% B for 4 minutes before the injection. The amino acids were automatically derivatized as follows: 5 µL of borate buffer pH 10.2 (Agilent, USA), 1 µL of sample/blank/analytical standard, 1 µL of a solution 1 mg/mL o-phataladehyde (Merck, Germany) in 2% (v/v) β-mercaptoethanol (Merck, Germany) or 2.5 mg/mL Fmoc chloride (Merck, Germany) in MeCN, 3 µL of a solution CH_3COOH 1 mol/L (Merck, Germany). The total injection volume was 10 µL. The elution was performed at a flow rate of 0.5 mL/min. The calibration curve for each amino acid (Merck, Germany) was made up in a range between 0.1 and 2 µg/mL. For the detection and quantification of the analytes, we mainly exploited the fluorescence detector. OPA-derivatized amino acids were excited at 338 nm and the emission signal detected

was 442 nm, while the Fmoc-derivatized one (proline) was excited at 262 nm and the emission was detected at 325 nm. As a further control, the UV detector was set at 338 nm for OPA derivatized amino acids, while proline was detected as 262 nm. The integration of the chromatograms was performed by using the Chromeleon Software (ThermoFisher, USA) and the concentration values obtained were normalized on the volume of sampled nectar in order avoid sampling biases and expressed as ng/ µL nectar. Total amino acids (TAA) content was calculated by adding the concentration of all the single amino acids, while the essential amino acids (EAA) content was obtained by adding the concentration of the amino acids essential for bees (Jeannerod et al., 2022).

Pollen phytochemicals extraction and analysis

The extraction and the subsequent quantification of secondary metabolites occurring in pollen samples was carried out as follows. One mg pollen for each sample was weighed and extracted in a ratio 1:1000 w/v in MeOH 70% v/v for two extraction cycles with the support of a bath sonicator (frequency: 37 Hz, temperature: 30°C). At the end of each extraction cycle, the supernatant was collected and dried under gaseous nitrogen (N_2). The extracts were resuspended in 1 mL H_2O and analysed for the total phenol (TPC) and flavonoid content (TFC) and the total antioxidant capacity (TEAC) as reported in Guzzetti et al., 2017 with minor

modifications. In detail, the quantification of flavonoids was made by using quercetin as analytical standard instead of catechin.

The extracts obtained from pollen samples were dried under gaseous nitrogen and analysed by HRMS for the untargeted metabolomic characterization. Both the negative and positive ionisation mode was considered for each sample analysed. The RP-HPLC-MS analysis was performed according to the same facilities and parameters provided in Pioltelli et al., 2023c. The chromatographic gradients were adjusted according to the profile occurring among the different species.

Statistical analysis

Data about pollen TPC, TFC, and TEAC and data on nectar TSC, TAA, and EAA were analysed to investigate putative response to landscape variables by using the software R (v. 4.3.1). Specifically, data on pollen TPC, TFC, and TEAC were normalised according to the min/max scaling method to account for the largely different ranges observed among the species investigated. We fitted Generalised Linear Mixed effects Models (GLMMs) with a binomial distribution of the response variable for data regarding the phytochemical composition of pollen. The dispersion parameter was monitored to be around 1 (in case of values lower than 1, the models were run with a beta distribution to avoid type II errors). The fixed effects included into the models were the land use variables obtained by the abovementioned PCA, naturally

uncorrelated, while the species were included as random components into the regression models. Data regarding nectar nutrition were analysed by GLMMs with a gaussian distribution of the response variable concerning TSC, while a Gamma distribution was implemented for TAA and EAA due to the violation of the assumption of normality.

To investigate inter-specific differences in the nutrients content among sampled species, we fitted (G)LM(M)s with the species as the fixed effect and the site as the random effect (in case of significant fluctuations of the nutrient of interest among sites). The dependent variables were assumed to be normally, or Gamma distributed for nectar nutrients and binomially (or beta distributed in case of overdispersion parameters < 1) for the analysis of the phytochemical composition of pollen.

The analysis of the metabolic profiles by HRMS was initially performed on the MS-DIAL software (Version 4.9) for peak peaking, deconvolution, noise setting and normalization. Normalized data were then analysed on R. Firstly, a Redundancy Analysis (RDA) was performed to test the impact of the land use variables (PC1 and PC2) on the overall metabolome of the pollen of the studied species. For the significant outputs, a random forest regression model was implemented to understand which the most significant metabolic features were responding to the land use variables. Packages exploited were TMB (Kristensen et al., 2016),

glmmTMB (Brooks et al., 2017), ggplot2 (Wickham, 2016), MuMIn (Bartòn, 2022), vegan (Oksanen et al., 2022), and rfPermute (Archer, 2022). The significant m/z were more deeply characterised by studying their fragmentation patterns in the MS/MS or DDA analysis both with literature research and using the UNIFI Software 1.9.4 EN (Waters, USA) with the library "Waters Traditional Medicine Library" provided by Waters, USA.

Results

Impact of the land use variables on nectar nutritional composition

The three analysed species presented differences in their nectar chemical profiles (Table S2). Concerning the analysis of nectar TSC in response to the landscape gradients, this was negatively influenced in a linear manner by the PC2 (Fig. 3) which describes a gradient moving from seminatural to agriculture dominated habitat (χ^2 = 3.985; p = 0.046). No significant effect of the urbanization gradient described by the PC1 was detected (χ^2 = 0.127; p = 0.722). The TSC of nectar did not vary significantly across the analysed species (Table S2).

No significant effects of the two land use gradients were observed on TAA (PC1 χ^2 = 0.162, p = 0.687; PC2: χ^2 = 0.017, p = 0.897) and EAA (PC1 χ^2 = 0.683, p = 0.494; PC2: χ^2 = 0.749, p = 0.454) content of nectar. A significant effect of the species on nectar TAA and EAA (Table S2) was observed.

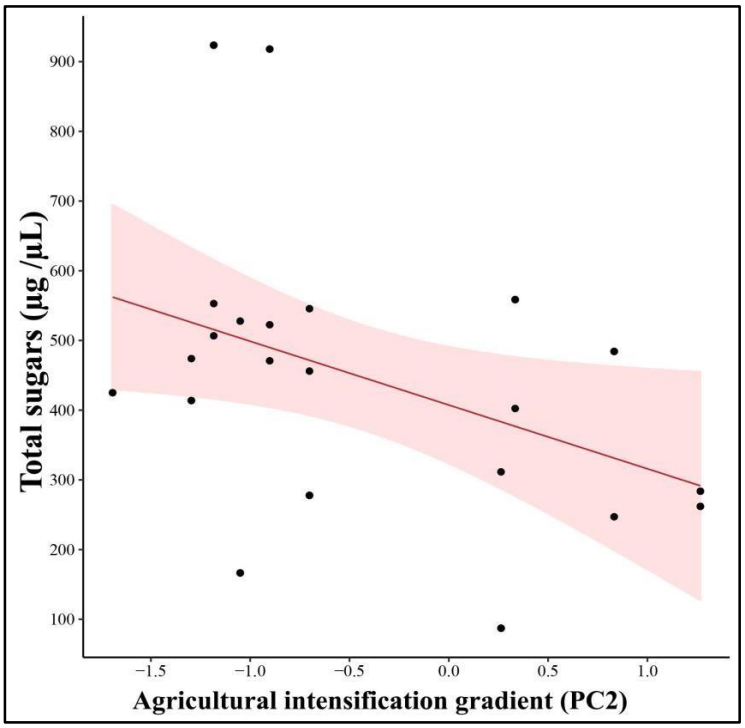

Figure 3. Relationship between the PC2 (defining an agriculture intensification gradient) and the TSC of nectars expressed as µg sugars per µL of sampled nectar (n = 22). The R2 value of the model is equal to 19.2%.

Impact of land use variables on pollen phytochemicals content

Among those analyzed, the species richest in phytochemicals was *P. reptans* followed by *L. corniculatus*, *H. radicata*, and *M. sylvestris*, as reported in Table S2. The phytochemical profile of pollen was not significantly influenced by the land-use variables concerning the total phenol content (PC1 $\chi^2 = 1.364$, $p = 0.245$; PC2: $\chi^2 = 1.804$, $p = 0.179$). However, the antioxidant activity of pollen showed a positive relation with the agricultural intensification gradient ($\chi^2 = 4.981$, $p = 0.026$) and the

total flavonoid content was positively related to the urbanization gradient ($\chi^2 = 3.86$, $p < 0.05$) as reported in Fig. 4a and 4b.

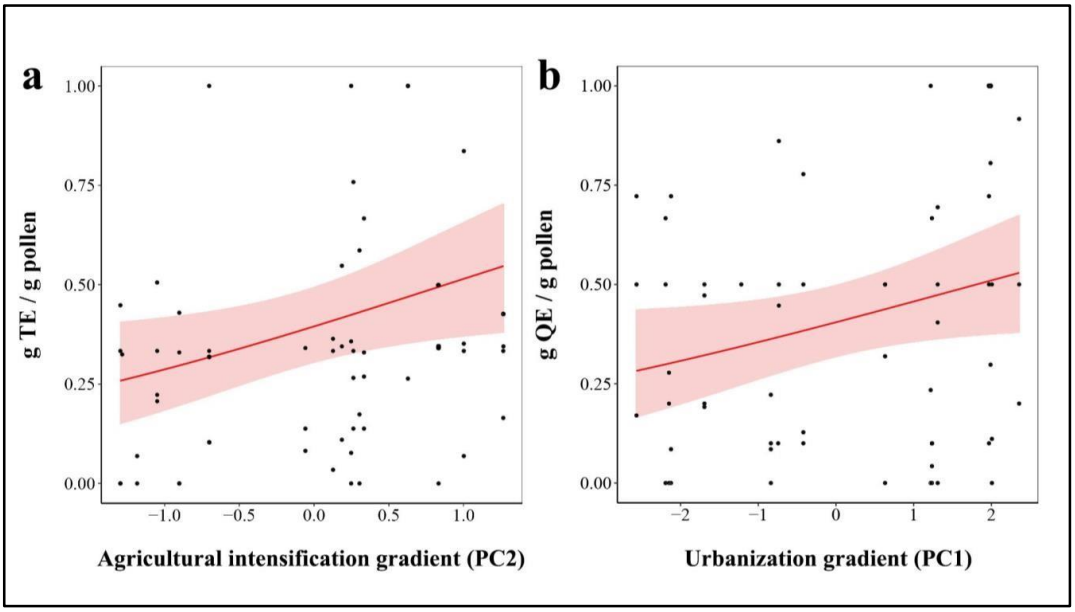

Figure 4. Relationship between the total phytochemicals content of pollen from the analyzed species and the landscape variables: (a) relation between the total antioxidant activity of pollen and the PC2 (n = 58; R2: 31.3%); (b) relation between the total flavonoid content of pollen and the PC1 (n = 58; R2: 31.7%).

Metabolomic assessment of pollen phytochemicals

The metabolomic analysis of pollen from wildflower species showed different responses among species to the land use variables. The output of the RDA is reported in Table 2 and 3. A significant response to the urbanisation gradient (PC1) was found in the metabolomic investigation of the pollen of *L. corniculatus*, both in positive and negative ion current, while the metabolome of *M. sylvestris* displayed

a significant response to the agriculture intensification gradient (PC2) in negative ionization mode.

Table 2. Output of the RDA considering the metabolomic analysis in negative ionisation mode.

Species	*P. reptans*		*H. radicata*		*L. corniculatus*		*M. sylvestris*	
Ion current	negative		negative		negative		negative	
Model	F	*p*	F	*p*	F	*p*	F	*p*
PC1	1.378	0.148	1.359	0.238	**2.474**	**0.021**	0.352	0.767
PC2	0.406	0.905	1.454	0.212	2.105	0.054	**9.097**	**< 0.001**

Table 3. Output of the RDA considering the metabolomic analysis in positive ionisation mode.

Species	*P. reptans*		*H. radicata*		*L. corniculatus*		*M. sylvestris*	
Ion current	positive		positive		positive		positive	
Model	F	*p*	F	*p*	F	*p*	F	*p*
PC1	1.261	0.286	1.939	0.08	**4.952**	**0.003**	1.95	0.136
PC2	0.39	0.799	0.975	0.422	1.051	0.389	2.733	0.066

Identification of the most responsive metabolites to land use variables

Phytochemicals most responsive to the urbanization gradient in *L. corniculatus* pollen were those belonging to the family of flavonoids (Table 4, also represented as the chromatographic traces in Fig. S1c, S2c, and S3c), and most of them was positively associated with the urbanization gradient described by PC1 (Fig. S1b). Additional information on the correlation coefficient between the land use variables (PC1 and PC2) and the significant features are reported in Fig. S1b, S2b and S3b. The analysis performed on *M. sylvestris* pollen extract shows an impact of the agricultural intensification gradient on the phytochemical composition of pollen, in particular a high degree of correlation was associated to an antioxidant compound, identified as rosmarinic acid (see Table 4). Additionally, Fig. S1a, S2a and S3a show the output of the random forest regression analysis and report the significant features responsible for the variation of pollen metabolome associated to land use variables in *L. corniculatus* (Fig. S1a, S2a) and *M. sylvestris* (Fig. S3a).

No significant effects of the land use gradient were detected in the metabolome investigation of *P. reptans* and *H. radicata* pollen, neither in negative nor in positive ionization mode.

Table 4. List of the identified metabolites resulted significant from the random forest analysis in the response to the land use variables. Rt = Retention time; m/z = mass/charge ratio.

Species	Peak	Adduct	Rt	m/z	MS/MS ions	Ontology	ID	Class	Ref
Lotus corniculatus	1	$[2M-Na]^+$	2.9	144	143, 128, 127, 117, 91	$C_{10}H_{14}N_2O_5$	Thymidine	Nucleoside	UNIFI
Lotus corniculatus	2	$[M-H]^+$	5.6	463	301	$C_{23}H_{26}O_{10}$	9,10-DiMP-3-O-Glc	Flavonoid	Zhang et al. 2015
Lotus corniculatus	3	[M-H]+	7.3	317	302, 285, 168, 158, 140, 134, 107	$C_{16}H_{12}O_7$	5,6,7,3'-Tetrahydroxy-4'-methoxyisoflavone	Flavonoid	UNIFI
Lotus corniculatus	4	$[M-H]^+$	7.5	301	286, 269, 241, 229, 153	$C_6H_{12}O_6$	5- methyl kaempferol	Flavonoid	UNIFI
Lotus corniculatus	5	$[M-H]^+$	8	549	301	$C_{26}H_{28}O_{13}$	9,10-DiMP-3-O-malonyl -Glc	Flavonoid	Zhang et al. 2015

Lotus corniculatus	6	$[M-H]^+$	10	331	316, 301, 298, 168	$C_{17}H_{14}O_7$	3,5,6-Trihydroxy-4',7-dimethoxyisoflavone	Flavonoid	UNIFI
Lotus corniculatus	7	$[M-H]^-$	5.6	387	369, 207, 163	$C_{18}H_{28}O_9$	Tuberonic acid glycoside	Hormone derivative	UNIFI
Lotus corniculatus	8	$[M-H]^-$	6	593	446, 430, 299, 285, 151	$C_{27}H_{30}O_{15}$	Kaempferol 3-O-L-rhamnopyranosyl-(1→2)-glucopyranoside	Flavonoid	UNIFI
Lotus corniculatus	9	$[M-H]^-$	7	649	503, 460, 446, 315, 313, 113	$C_{31}H_{38}O_{15}$	Tubuloside E	Phenylpropanoid	UNIFI
Lotus corniculatus	10	$[M-H]^-$	7.2	463	301, 286	$C_{22}H_{24}O_{11}$	Hesperetin-7-O-glycoside	Flavonoid	UNIFI
Lotus corniculatus	11	$[M-H]^-$	10	329	229, 211, 183, 171	$C_{18}H_{34}O_5$	Sanleng acid	Fatty acid	UNIFI

Lotus corniculatus	12	$[M-H]^-$	12	982	941, 923, 879, 615, 597, 247, 205, 163, 139	$C_{49}H_{80}O_{20}$	Agroastragaloside IV	Saponin	UNIFI
Malva sylvestris	13	$[M-H]^-$	0.8	282	150, 133, 108	$C_{10}H_{13}N_5O_5$	Guanosine	Nucleoside	UNIFI
Malva sylvestris	14	$[M-H]^-$	3	359	197, 153, 123	$C_{18}H_{16}O_8$	Rosmarinic acid	Phenolic acid	UNIFI
Malva sylvestris	15	[M-H]-	5.4	655	493, 330	$C_{28}H_{22}O_{18}$	Patuletin diglucoside	Flavonoid	Boukhris et al. 2016

Discussion

The first important result of this study is the identification of a well-marked relationship between landscape composition and some aspects of the chemical profile of both pollen and nectar. The total sugar content of the nectar was negatively influenced by the agriculture intensification gradient, while no significant variations in nectar TAA and EAA were detected in response to any of the land use gradients considered. It is conceivable an effect of agrochemicals (i.e., herbicides) in the surrounding of the crops which may alter sugar metabolism, since many herbicides commercially available impair the photosynthesis efficiency (Oettmeier, 2003). However, a recent study by Russo et al., 2023 on a different panel of flowers did not find any significant variation on nectar sugars concentration based on the herbicide treatment, making this topic in need of further investigation, hopefully on a wider phylogenetic set of plants.

Concerning the phytochemical composition of pollen, secondary metabolites are gaining increasing attention due to the important nutraceutical properties that some of them exhibit, for instance by acting as pesticide detoxifiers or by reducing the probability of parasite infections upon specific dosages (Đorđievski et al., 2023; Riveros & Groenenberg, 2022; Mao et al., 2013). In this study we found that the overall antioxidant activity of pollen increases significantly along the agriculture

intensification gradient, while along the urbanization gradient a specific increase in the flavonoid content was observed. It is arguably that these patterns may be explained by the higher stress levels experienced by the plants. The transition from natural to urban areas is likely to pose some pressures to plants, for instance by increasing the transpiration level due to higher temperature and lower humidity which may elicit the biosynthesis of stress-defence compounds, such as flavonoids (Qian et al., 2022; Innes et al., 2019), also at the pollen level (Rutley et al., 2021), as highlighted in the present study. Many flavonoids have recently been appointed as nutraceutical in the diet of pollinator insects (Riveros & Groenenberg, 2022; Fitch et al., 2022) and a higher occurrence in the pollen of species growing in urbanized areas may be helpful in mitigating stress phenomena, such as those related to oxidation and ageing (Berenbaum & Calla, 2021). Concerning the positive correlation observed between the pollen antioxidant activity and the agriculture intensification gradient, the occurrence of agrochemicals in agricultural areas (mainly herbicides) could act as a driver of stress for wild plants triggering the production of defence compounds (Cesco et al., 2021). Another important result of our study derived from the metabolomic investigations on the pollen extracts was the identification of the species more responsive to the land use gradient. We found that the chemical composition of the pollen of *L. corniculatus* was significantly impacted by the urbanization gradient, whilst the pollen of *M. sylvestris* responded

to the agriculture intensification one. The most important metabolites influenced by the land use variables belong to the class of phenolics, mainly flavonoids, many of which were positively related to the PC1 in the pollen of *L. corniculatus*. However, this pattern was not associated with an increase in the antioxidant activity nor in the total phenol content of pollen, and this may be because some phenolic compounds other than flavonoids or belonging to different classes of phytochemicals may balance the flavonoids variation. Indeed, in the pollen of *L. corniculatus* we found that some phytochemicals highlighted by the random forest analysis were negatively associated with the PC1. Concerning the agriculture intensification gradient, the increase in the total antioxidant activity of pollen may be partly related with the metabolomic variations occurring in the pollen of *M. sylvestris*. We found that rosmarinic acid, a potent antioxidant compound (Chadni et al., 2023), was positively related to the PC2 and may be involved in the total antioxidant activity observed from pollen extracts. However, another identified compound endowed with antioxidant properties, a patuletin derivative, showed a negative correlation with the PC2. Nevertheless, the radical scavenging properties of the different metabolites may vary depending on their exact concentration which was not evaluated in the present research.

In a context where landscape anthropization can influence the chemical composition of floral rewards at different metabolic levels, providing an effective

plant community able to sustain the diet of pollinator insects should be considered for the planning and definition of effective Nature based Solutions (NbS) in highly anthropized environments. In recent years, NbS are particularly attracting the interest of policy makers due to their potential contribution to biodiversity protection and human well-being (Lafortezza et al., 2018). However, their reliability at the plant-pollinators interaction level is still poorly evaluated and requires disentangling which plant species must be prioritised to support the trophic needs of pollinators. In the present study, we found a higher TAA and EAA content in the nectar of *T. pratense* compared with *P. vulgaris* while no significant differences were shown in the sugar content among the studied species (Table S2). Previous studies have already suggested that the nectar of species belonging to the Fabaceae family represent an important source of amino acids (Gardener & Gillman, 2001) whose content is an important factor influencing the foraging preference of pollinators (Venjakob et al., 2020). Conversely, the phenolic composition of pollen and consequently the related antioxidant activity was significantly higher in *P. reptans*, followed by *L. corniculatus*, and ultimately *H. radicata* and *M. sylvestris* (Table S2). However, the secondary metabolites composition needs to be specifically defined since not all of them exert beneficial effects in the diet of pollinators. For instance, tannins are one of the major constituents of the phytochemical composition of *P. reptans* (Tomczyk & Latté, 2009) and may act as anti-nutrients, affecting the

longevity of bees (Sagona et al., 2021). In the present study, our focus was posed on the small molecules and phytochemicals composition of flower rewards, since these compounds, differently from macronutrients (e.g., proteins and lipids), are the most frequently involved in short-term responses to changes in environmental variables, acting as osmolytes or defence/stress compounds (Egan et al., 2021; Ghosh et al., 2021; Arathi et al., 2018). Furthermore, these compounds are known to play a significant role in pollinators' diet, acting as essential nutrients or as nutraceuticals (Koch et al., 2017; Richardson et al., 2015). The integration of these results with those arising from the investigation of the nutritional profiles of foraged pollen may be of help to disentangle at a deeper scale the effect of land use management on plant-pollinators trophic interactions.

It is important to highlight that local abiotic factor, such as solar radiation, temperature, and evapotranspiration might display great effects on the relative content of sugars in nectar (Plos et al., 2023), and polypeptides in pollen (Descamps et al. 2021). The magnitude of these microclimatic variables may be relevant, especially concerning short-term variations in the concentration of small molecules like those analysed in the present study (Qian et al., 2022; Innes et al., 2019). Such modifications may even impact floral traits and pollinator efficiency (Akter & Klečka., 2022). At the individual level, the genotype, and the stage of development of the flower may impact significantly on the chemical composition of nectar

(Clearwater et al., 2018). For instance, the content of sugar in nectar is very low at the first floral stage, then it increases up to a peak at the second stage and then it tends to decline during the third floral stage (Clearwater et al., 2018). Furthermore, also the sexual floral stage was found to significantly impact the chemistry of nectar: for example, in *Echium vulgare*, the nectar sugar content is significantly higher during the female phase compared to the male one, while the content of phenylalanine was shown to be significantly higher during the male stage than the female one (Barberis et al., 2021). Although these factors acting more at the individual- or even flower-level were not quantified in our study, they likely constitute a strong source of variation deserving future scientific insights.

From the applicative point of view, this investigation provides some practical implications for the management of urban and peri-urban green areas and the design of ecological restoration strategies in human-dominated landscapes. For example, the reduction in the sugar content of nectar observed in agricultural areas has strong implications for the potential of these sites to sustain the local pollinator communities. Indeed, if we convert the concentration of sugars observed in energetic terms (J/µL) and considering that an average 500 mg bee requires 600 J per hour of flight (Wilmer et al., 2011), we can calculate that this representative energetic requirement can be satisfied by foraging 11 µL nectar in agricultural areas while only 5 µL in semi-natural areas. Thus, the halving of the sugar content along

the agriculture intensification gradient suggests the risk to increase the time devoted to foraging for pollinator insects. This result highlights the critical importance of boosting flower availability in these habitats to sustain the energetic needs of the local insect pollinators community. A more detailed study of the environmental variables influencing sugar content could also make it possible to plan tailored interventions across different areas, allowing for example a more nuanced control over the application of herbicides and other management actions. The increase in the flavonoids and antioxidant compounds in disturbed areas poses the attention on the impact of anthropogenic stressors on plant secondary metabolism. As previously highlighted, many of these metabolites have shown promising bioactivity against pesticides and parasites at the physiological level, which hints at a potential avenue for ecological fortification. However, it is essential to exercise caution, given that many of these compounds could exceed toxicity thresholds (Palmer Young et al., 2019). Then, the seedling of species characterized by higher resilience to anthropogenic stressors in terms of secondary metabolism variations emerges as a prudent course of action. This approach can mitigate the potential hazard of exposing pollinator insects to toxic concentration of phytochemicals. Furthermore, providing for a diversified panel of plant species in a long lasting seasonal context can be crucial as a rich plant community affords

pollinators the flexibility to selectively exploit resources based on their foraging needs and preferences (Blüthgen and Klein, 2011).

Conclusions

With this study we have deepened some aspects dealing with the nutritional ecology of pollinators focusing specifically on the resources they forage on. The results highlighted that some variations occurring in bee pollen nutrients and related to different land use managements could not be only due to the choice of foragers, but also depend on the plant resource itself. The variations highlighted in the chemical composition of pollen and nectar may impact the health status of pollinator insects in different environments, attenuating and/or exacerbating stress phenomena occurring at the insect physiological level. The present study suggests that the definition of a proper nutritional landscape for pollinator insects requires not only to identify the most nutritionally relevant species for pollinators, but also to understand to which extent habitat anthropization may modify their nutritional uptake by acting on plant metabolic pathways, in order to promote reliable actions for the planning of mitigation strategies in urban contexts, such as the NbS, by selecting species able to offer a proper nutrient composition regardless of the environment in which they occur.

ACKNOWLEDGMENTS

The authors thank Fausto Ramazzotti, Nicola Tommasi, Andrea Decimi, and Luca Tonietti for their help with the sampling activities, Maura Brioschi and Simona Serio for their support with the mass spectrometry and amino acids analyses, respectively.

Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU,Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP, H43C22000530001 Project title

“National Biodiversity Future Center - NBFC”. PB acknowledges support from Italian Ministry of Universities and Research (with resources from the PONRI FSE REACT-EU 2014–2020 – “Azione IV.4 - Dottorati e contratti di ricerca su tematiche dell'innovazione, Azione IV.6 - Contratti di ricerca su tematiche Green”).

DECLARATION OF COMPETING INTEREST

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

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APPENDIX A

Text S1- Land use characterization

Landscape composition was characterized in a buffer area of 1 km around each sampling site and based on the DUSAF 6.0 land-use cartography. (2018 - DUSAF 6.0; https://www.dati.lombardia.it/Territorio/Dusaf-6-0-Uso-del-suolo-2018/7rae-fng6 accessed 26/06/2021)

This map is available at a scale of 1: 10.000 with a minimum linear dimension of polygons of 20 m and was developed from AGEA orthophotos and SPOT 6/7 satellite images. The original level and sub-level of land-use classification were grouped in 3 main land-use classes.

In detail: DUSAF land use level type "11231", "1411", "1412", "2115", "2242", "2311", "2312", "314", "31111", "31121", "3113", "3222", "3241", "3242", "411" were categorized as "Semi-natural"; DUSAF land use level type "124", "131", "132", "133", "134", "1111", "1112", "1121", "1122", "1123", , "12124", "1221", "1222", "12111", "12121", "12122", "12123", "12125", "12126" were categorized as "Impervious cover"; DUSAF land use level type "213", "221", "222", "223", "2111", "2112", "2241", "2242", "12112" were categorized as "Agricultural fields".

(Full explanation of codes is available at https://www.cartografia.regione.lombardia.it//metadata/Dusaf/doc/Legenda_DUSAF_2018_6_0.pdf)

Table S1- Land use characterization

The table reports the full description of DUSAF 6.0 land use classification along with their categorization into the 3 main classes made by the authors.

DUSAF description	Category
1111 - Residential dense fabric	Impervious
1112 - Continuous moderately dense residential fabric	Impervious
1121 - Discontinuous residential fabric	Impervious
1122 -Sparse and nucleiform residential fabric	Impervious
1123 - Sparse residential fabric	Impervious
11231 - Farmhouses	Semi-natural
12111 -Industrial, craft, and commercial settlements	Impervious
12112 - Agricultural settlements	Agricultural
12121 -Hospital facilities	Impervious
12122 -Public and private service facilities	Impervious
12123 - Technological facilities	Impervious
12124 - Cemeteries	Impervious
12125 - Obliterated military areas	Impervious
12126 - Ground-mounted photovoltaic systems	Impervious
1221 - Road networks and ancillary spaces	Impervious
1222 - Railway networks and ancillary spaces	Impervious
124 - Airports and heliports	Impervious
131 - Quarries	Impervious
132 - Dumpsites	Impervious
133 - Construction sites	Impervious
134 - Undeveloped and non-vegetated degraded areas	Impervious
1411 - Parks and gardens	Semi-natural
1412 - Uncultivated green areas	Semi-natural
2111 - Plain farmlands	Agricultural
2112 - Wooded arable lands	Agricultural
2115 - Backyard gardens	Semi-natural
213 - Rice fields	Agricultural
221 - Vineyards	Agricultural
222 - Orchards and minor fruits	Agricultural
223 - Olive groves	Agricultural
2241 - Poplar plantations	Agricultural
2242 - Other agrarian woody species	Agricultural
2311 - Permanent grasslands without tree and shrub species	Semi-natural
2311 - Permanent grasslands with tree and shrub species	Semi-natural
31111 - Medium to high-density hardwood forests managed as coppices	Semi-natural
31112 - Medium to high-density hardwood forests managed as high forests	Semi-natural
31121 - Low-density hardwood forests managed as coppices	Semi-natural
3113 - Riparian ecosystems	Semi-natural
314 -Recent reforestations	Semi-natural
3222 - Riparian vegetation	Semi-natural
3241 - Thickets with significant presence of tall shrubs and trees	Semi-natural
3242 - Bushes in abandoned agricultural areas	Semi-natural
411 - Vegetation of inland wetlands and peat bogs	Semi-natural

Table S2. Pollen and nectar chemistry

The table reports the detail on the chemical composition of nectar and pollen of the studied species.

Data are expressed as a concentration for the nectar and as a weight ratio for the pollen. The uppercase letters indicate the significance of the comparison of the nutritional and phytochemical content among the studied species. Different letters in the same nutritional categories indicate a difference significant at the statistical level ($p < 0.05$). Data are reported as the mean ± SEM.

Species	Total Sugars (µg/ µL)	TAA (ng/ µL)	EAA (ng/ µL)
Trifolium pratense	593.42 ± 63.61[a]	288.02 ± 9.89[a]	46.02 ± 2.43[a]
Prunella vulgaris	310.56 ± 48.33[a]	16.26 ± 0.48[b]	2.35 ± 0.14[b]
Salvia pratensis	420.43 ± 55.98[a]	91 ± 5.53[a]	28.32 ± 4.47[a]

Species	TPC (µg GAE/ mg)	TEAC (µg TE/ mg)	TFC (µg QE/ mg)
Potentilla reptans	173.1 ± 101[a]	284.5 ± 1.94 [a]	42.79 ± 0.26[a]
Hypochaeris radicata	11.54 ± 0.15[b]	9.68 ± 0.19[b]	3.15 ± 0.07[b]
Lotus corniculatus	48.3 ± 0.80[c]	52.98 ± 0.65[c]	23.18 ± 0.34[c]
Malva sylvestris	11.18 ± 0.19[b]	0.77 ± 0.02[d]	0.71 ± 0.01[d]

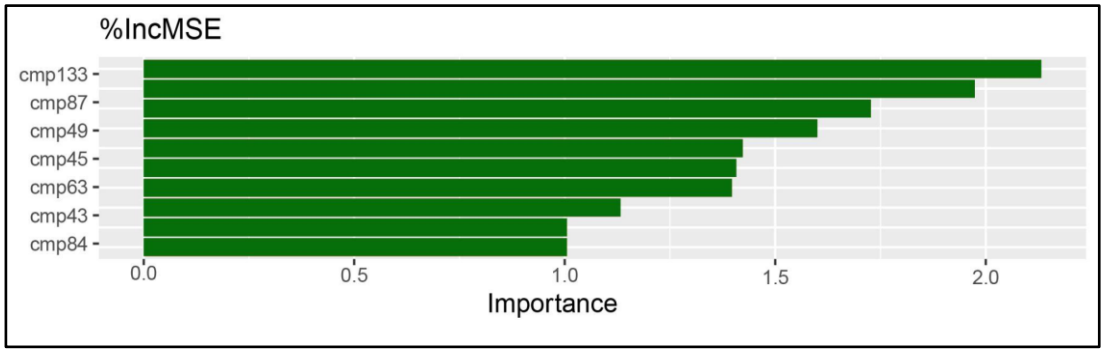


Figure S1a - Output of the random forest regression analysis performed on the significant features belonging to the positive ionizing metabolome in *L. corniculatus* pollen extracts responding to the PC1 (urbanization gradient). Each code is a different compound.

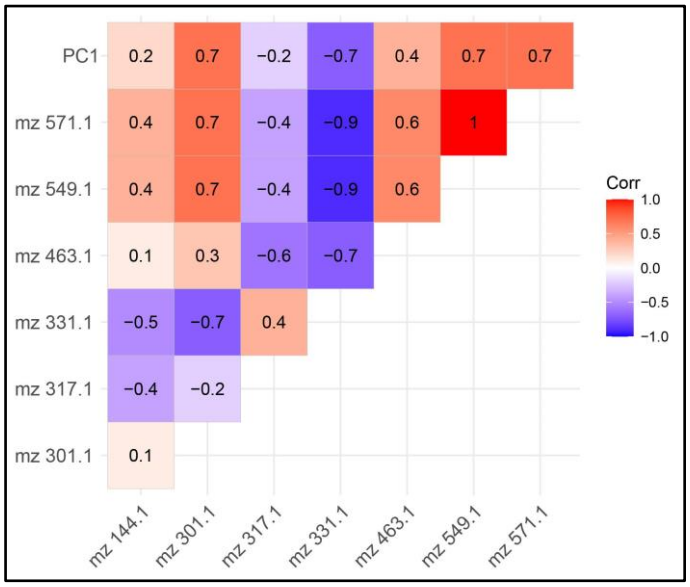


Fig. S1b - Correlation plot showing the relationship between the PC1 and the most relevant metabolites in positive ionizing mode for *L. corniculatus* pollen extracts. Each code is a different compound.

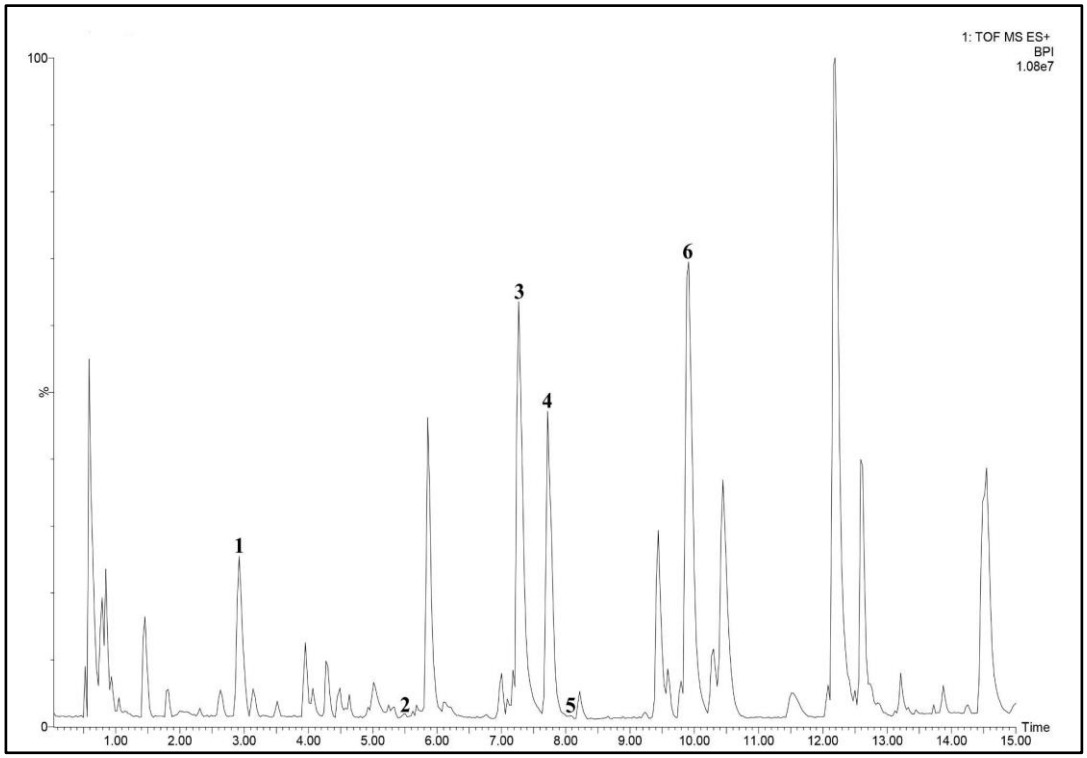


Fig. S1c - BPI chromatogram of the fullscan trace in positive ionization mode of *L. corniculatus* pollen extract. The numbers refer to the features significantly related to the PC1 and characterized in Table 2.

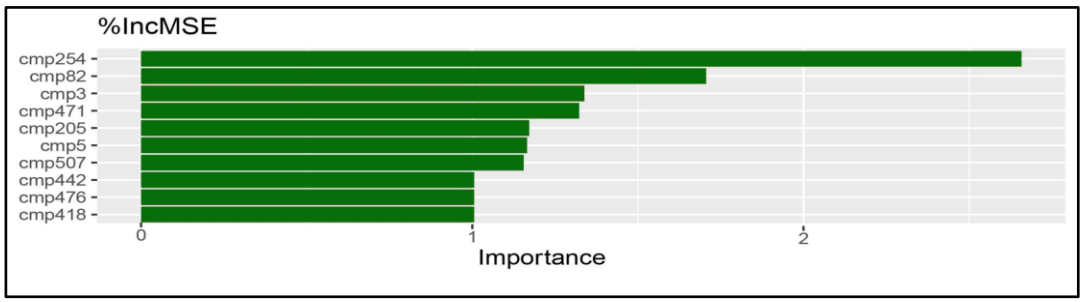


Fig. S2a: Output of the random forest regression analysis performed on the significant features belonging to the negative ionizing metabolome in *L. corniculatus* pollen extracts responding to the PC1 (urbanization gradient). Each code is a different compound.

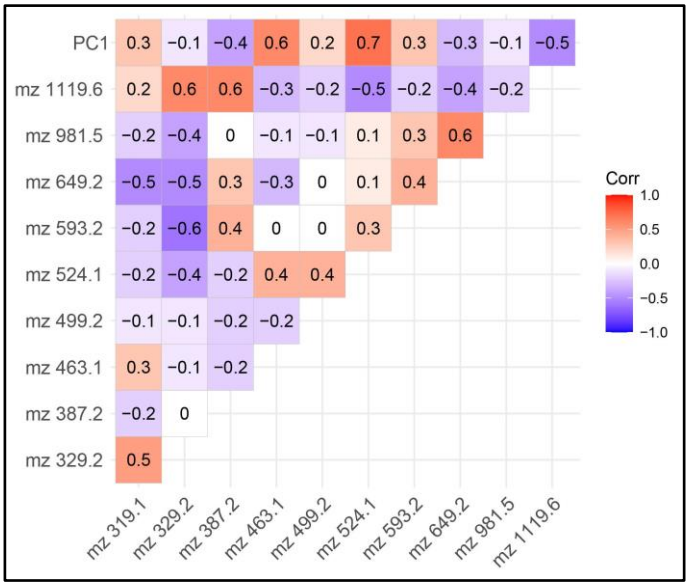


Fig. S2b: Correlation plot showing the relationship between the PC1 and the most relevant metabolites in negative ionizing mode for *L. corniculatus* pollen extracts. Each code is a different compound.

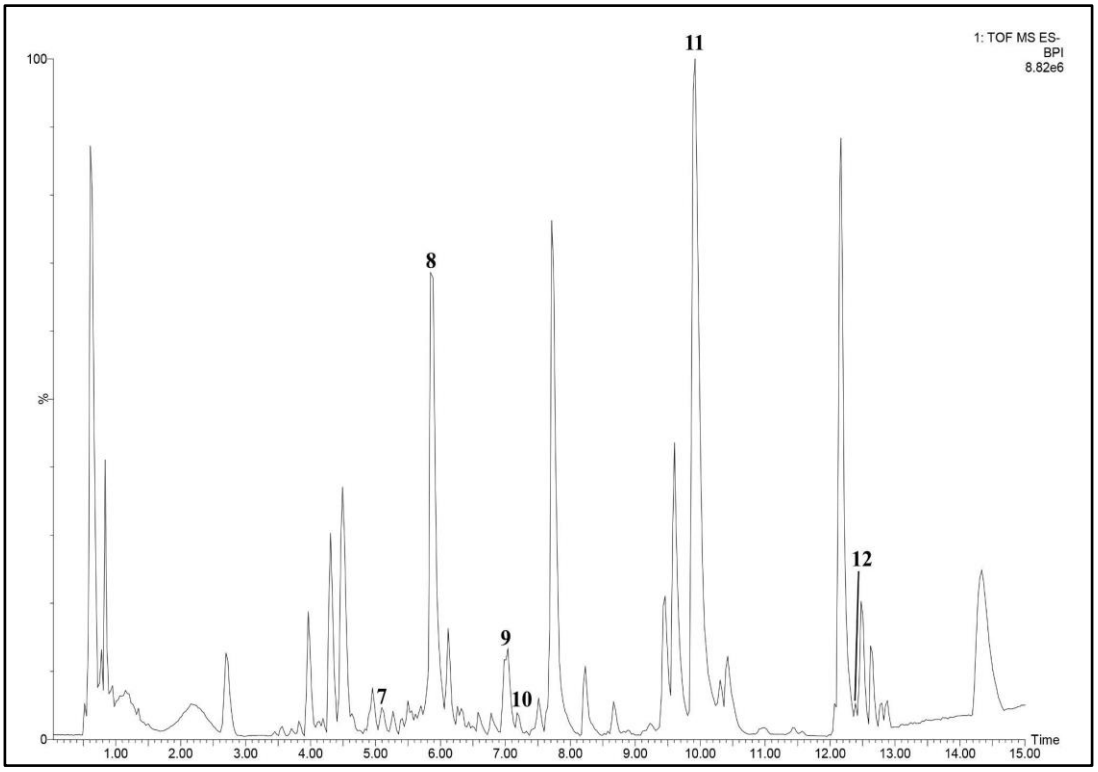


Fig. S2c: BPI chromatogram of the fullscan trace in negative ionization mode of *L. corniculatus* pollen extract. The numbers refer to the features significantly related to the PC1 and characterized in Table 2.

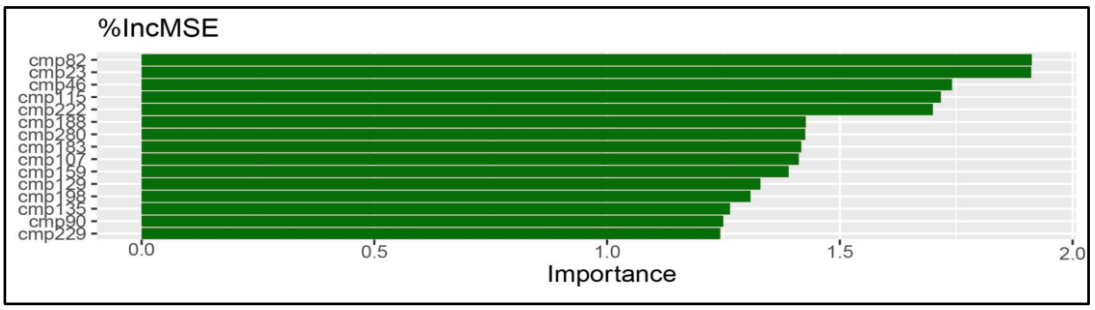


Fig. S3a: Output of the random forest regression analysis performed on the significant features belonging to the negative ionizing metabolome in *M. sylvestris* pollen extracts responding to the PC2 (agricultural intensification gradient). Each code is a different compound.

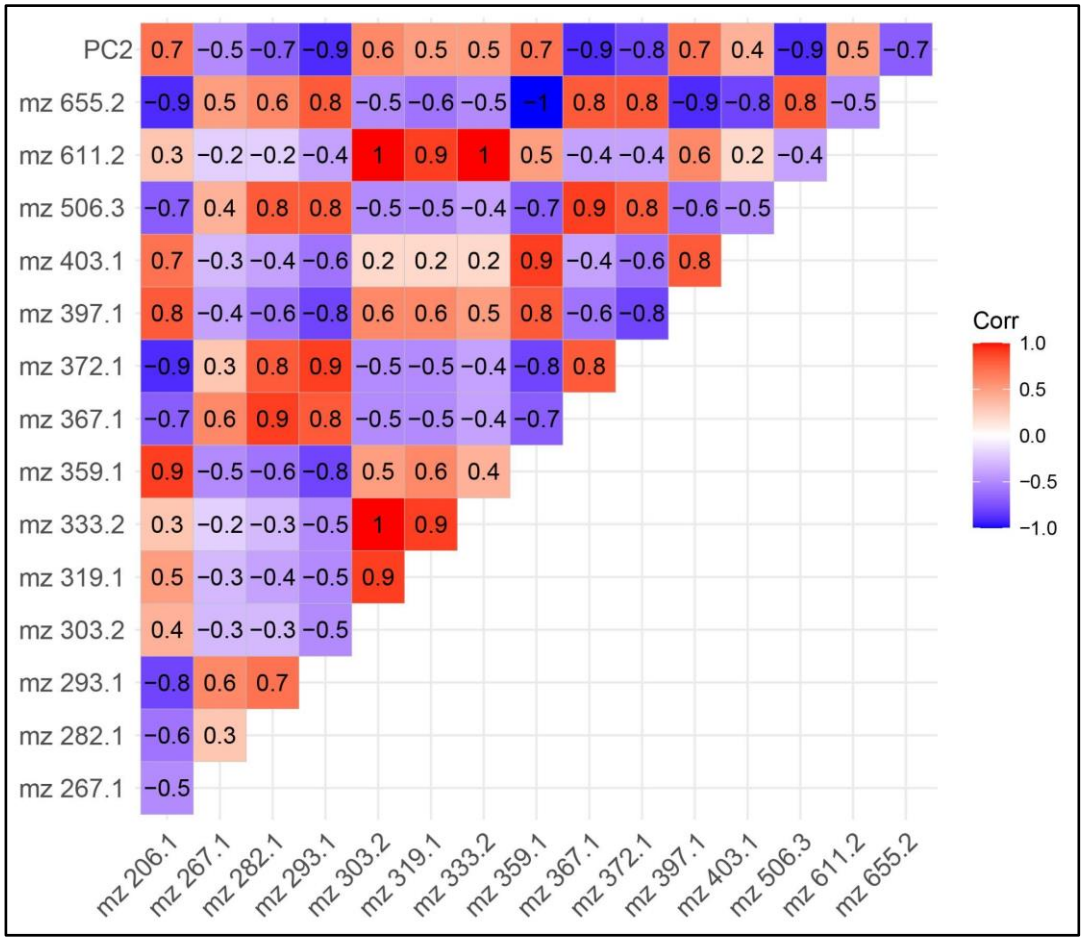


Fig. S3b: Correlation plot showing the relationship between the PC2 and the most relevant metabolites in negative ionizing mode for *M. sylvestris* pollen extracts. Each code is a different compound

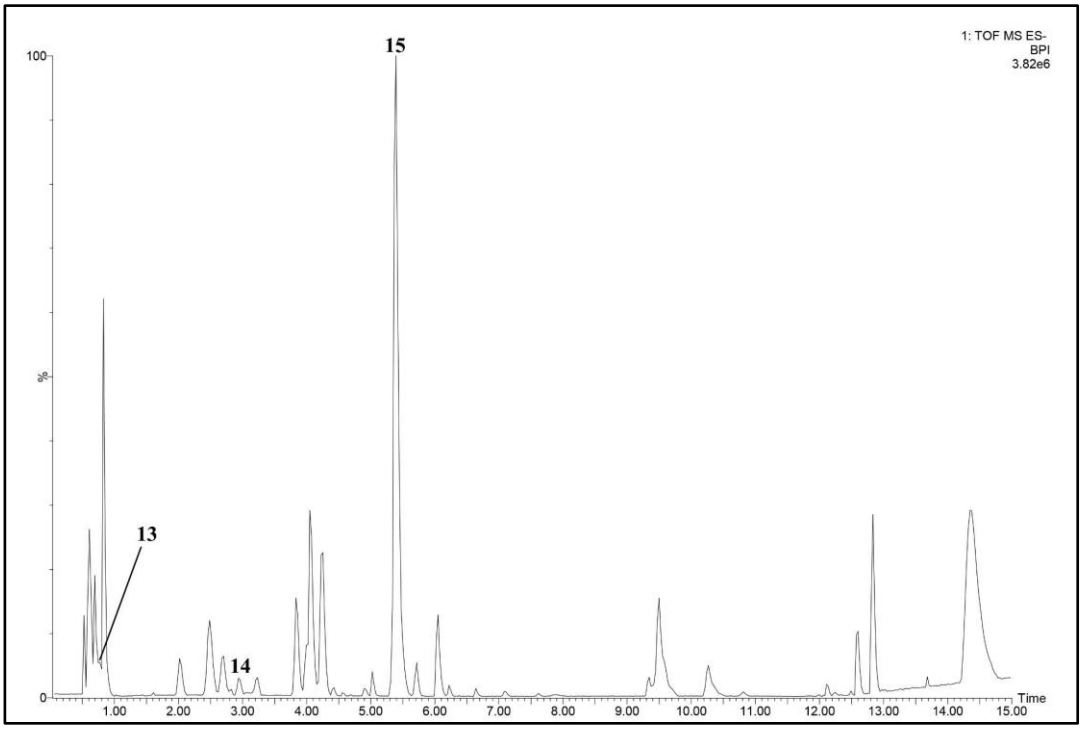


Fig. S3c: BPI chromatogram of the fullscan trace in negative ionization mode of *M. sylvestris* pollen extract. The numbers refer to the features significantly related to the PC2 and characterized in Table 2.

Chapter II

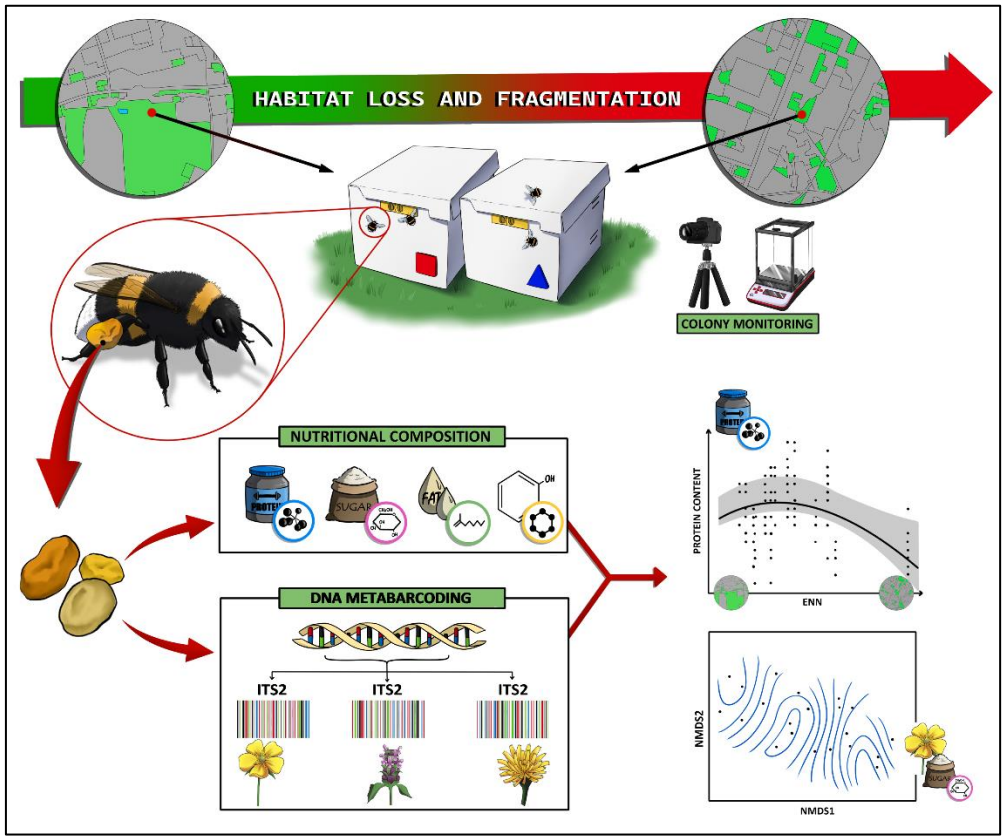
Influence of habitat fragmentation on pollinators' nutritional ecology

Case Study IV. Pioltelli, E., Guzzetti, L., Ouled Larbi M., Labra M., Galimberti A. & Biella P. (2023). Landscape fragmentation constrains bumblebee nutritional ecology and foraging dynamics.

Type of article: Research paper

Status: Under review in "*Landscape and Urban Planning*""

Graphical abstract



Landscape fragmentation constrains bumblebee nutritional ecology and foraging dynamics.

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KEYWORDS: Bee; Plant-pollinator interactions; Pollinators diet; Pollen DNA metabarcoding; Urban greening; Habitat fragmentation

Abstract

Habitat fragmentation is modifying landscapes and the distribution of floral resources, possibly shaping pollinator resource acquisition, which is an issue of global concern for pollinator health and urbanization sustainability. Here, using urban parks as field laboratories for the dramatic contrast around, we aimed to clarify how fragmentation and local flower availability shape bumblebee foraging dynamics by characterizing several components: the nutritional content and plant composition of collected pollen pellets, the foraging rate, and the plant-nutrition association along a fragmentation gradient. We found mostly negative linear or non-linear relationships between nutritional quality and fragmentation, tight plant composition-nutrition associations interpretable as low access to alternative resources, and shorter foraging time in smaller green areas, showing behavioural limits by the landscape. Thus, fragmentation can constrain all aspects of bumblebee foraging by compromising resource accessibility. This study illuminates the link between landscape features and the nutritional ecology of pollinators, a key aspect for understanding pollinator foraging dynamics. The findings of the study can provide valuable guidelines for policy maker and stakeholders involved in the management and ecological restoration planning for urban green areas and even for outlining mitigation measures in urban context.

Introduction

The increase in the impervious cover associated with urbanization leads to the worldwide loss and fragmentation of suitable habitats that provide nesting and food resources to pollinators (Biella et al. 2022), which may impact the way pollinators forage. These changes in landscape composition and configuration inevitably result in lower connectivity between green patches (Wenzel et al. 2020). Along with the decline in connectivity, the size reduction of green fragments can decrease their quality intended as their potential to sustain local populations of pollinators (Fahrig 2003). These conditions affect both the plant and pollinator communities' structure and their interactions (Grass et al. 2018) with important implications for pollinator's nutritional ecology and for the related ecosystem service as well (Gervais et al. 2020; Theodorou et al. 2020). The fragmentation of green areas in landscapes may shape multiple aspects that influence the nutritional ecology of pollinators (Hülsmann et al. 2015; Winfree et al. 2011), such as the availability in terms of richness and abundance of floral resources (Potts et al. 2010) and their spatial distribution (Matteson et al. 2013). For instance, fragmentation can lead to the dishomogeneous distribution of plant resources to pollinators, as indicated by the linear decrease of pollen diversity collected by pollinators across a gradient of green areas fragmented by urbanization (Biella et al. 2022). Furthermore, fragmentation can directly influence the foraging behaviour of pollinators (Gervais et al. 2020). According to the optimal foraging theory, pollinators will forage closer to their nesting in a landscape characterized by a lower degree of habitat fragmentation and an even distribution of resources to reduce their energetic expenditure (Goulson 1999). This was confirmed by a study showing that landscapes with higher green coverage were associated with shorter foraging distances and trip duration in several bumblebee species (Redhead et al. 2016).

Therefore, the spatial limitations imposed by landscape configuration on pollinator foraging behaviour, coupled with the consequent decline in the availability and quality of the resources, can act synergically, ultimately jeopardizing urban pollinators population conservation.

Eusocial pollinators, such as bumblebees, characterized by colony life cycles that last for several months (Crone & Williams 2016), are particularly susceptible to dishomogeneity in the nutritional landscape as they benefit from having continuous access to floral resources to sustain colony growth (Couvillon et al. 2014). Indeed, bumblebee fitness appears as symmetric to the seasonal distribution of flower resources and nutrients: for instance, late-season shortages are associated with fewer bees the following year (Timberlake et al. 2020). Moreover, local features (e.g., floral resources availability and quality) and landscape scale drivers (e.g., amount and fragmentation of green patches) are intimately related to bumblebee colony health, performance, and reproductive success (Theodorou et al. 2022; Vaudo et al. 2018). Previous studies on *Bombus terrestris* observed an increase in the colony growth rate and in the total number of workers at sites characterized by a higher diversity of floral resources (Goulson et al. 2002) and a reduction of colony fitness in highly urbanized areas (Theodorou et al. 2022). These negative effects on colony performance can be mainly ascribed to an unbalanced assumption of macronutrients (i.e., proteins, lipids, carbohydrates) and phytochemicals and especially to a low quality of the pollen provision that represents a primary food source for the larvae (Kriesell et al. 2017; Nicolson et al. 2018).

The importance of a balanced assumption of macronutrients and phytochemicals has been emphasized by evidence from several bees showing clear food choices of floral resources to meet their nutritional demands (Kriesell et al. 2017; Liu et al. 2006; Ruedenauer et al. 2016; Vaudo et al. 2016). Pollen macronutrients play a key role in this food choice as indicated by

experiments showing that *B. terrestris* and *B. impatiens* workers can regulate their protein and lipid intake preferring pollen with higher protein concentration while avoiding provisions too rich in lipids (Vaudo et al. 2016). Beyond single nutrient concentrations, the ratios of macronutrients are also pivotal to describing bees foraging behaviour, such as the protein:lipid ratio (P:L) (Vaudo et al., 2016). However, most of these studies were either in laboratory-controlled conditions or across permeable landscapes (such as semi-natural to agricultural areas); still, little is known about pollinators' nutritional ecology across a gradient of severe fragmentation where green areas are dispersed in highly inhospitable, cemented zones.

Here, we developed a field-based study to clarify how the amount and fragmentation of green areas of urban landscapes and the local availability of flower resources shape the foraging dynamics and the nutritional intake of a social pollinator. Two main expectations could be drawn regarding how bumblebee foraging could respond to fragmented landscapes. First, patchy dispersed green areas could decrease the nutritional quality of the pollen brought to the colony if plant species diversity at foraging sites is low. This is supported by a scenario in which the foragers encounter strong spatial constraints in patch distribution and quality that prevent access to equally profitable resources across landscapes (Barraquand & Benhamou, 2008). In this case, we would also observe a tight association between plant taxonomic composition of pollen pellets and the chemical profiles of collected resources, indicating little use of alternative plant sources (Biella et al. 2019). Secondly, bumblebee colony behaviour in terms of the rate of workers leaving the nest for foraging could reflect the heterogeneous distribution of resources, for instance by foraging longer in areas with dispersed resources by maximizing quantity over quality or increasing searching time (Jha & Kremen 2013; Weterings et al. 2018). It is also important to notice that the foraging frequency of bee workers reflects the foraging effort of a colony needing resources (e.g., Biella et al 2019,

Pernal & Currie 2001). To test these expectations, we aimed to (i) characterize the chemical composition of pollen pellets and their specific plant composition in landscapes of varying fragmentation (size, isolation of green areas), (ii) evaluate the association between plant composition and pollen nutrients in the resources brought to the colony, (iii) describe changes in the frequency of foraging to search for behavioural adjustments to the fragmented landscape. This study highlights the performance of varying fragmented landscapes in sustaining pollinators by evaluating several components that ultimately determine pollinator nutritional ecology: food preferences in terms of chemistry and diversity of collected flower resources and colony behavioural responses as foraging rate. Our study provides an empirical and multicomponent characterization of how landscape and local features interact in shaping the nutritional ecology of key pollinators, that could even be used as a basis for outlining reinforcement measures for pollinators in urban contexts.

Materials and methods

Study design and sampling

Study sites were chosen to represent a gradient of different green areas size and fragmentation within an urban landscape, although all sites were the same type of habitat, namely green areas accessible to the public for recreational purposes (e.g., urban parks). The mean distance between sites was 2.5 km (min. = 1.2 km; max. = 4.3 km) to avoid spatial non-independence as this distance is above the usual foraging range observed for the selected pollinator model *Bombus terrestris* (Redhead et al. 2016; Wolf & Moritz, 2008). This species is common in Europe and can be easily found in different habitats (Polce et al. 2018), even in urban areas (Banaszak-Cibicka & Żmihorski 2012; Meeus et al. 2021). A pair of commercial

colonies of this species (acquired from Bioplanet, Forlì-Cesena, Italy) was placed at each of 14 sites in different parts of the city of Milan, in Northern Italy (Figure 1). Colonies were elevated from the ground and protected from rain and sun with wooden sheds covered with an insulating sheet (Figure 1). Each colony was left at each site for at least 48 hours. Most of the sites were sampled twice, at a month time of distance using a different colony set and only if the plant community was very different from the previous sampling round (after a detailed visual investigation); Thus, the final number of sampling events was 29 and the samplings lasted from May 27th to July 30th, 2020.

To evaluate if 48h time was representative or robust against possible bumblebee life cycle variations, we measured the weight of bumblebee colonies and the plant richness of the pollen transported in a subset of 4 sites that were weekly sampled over a month.

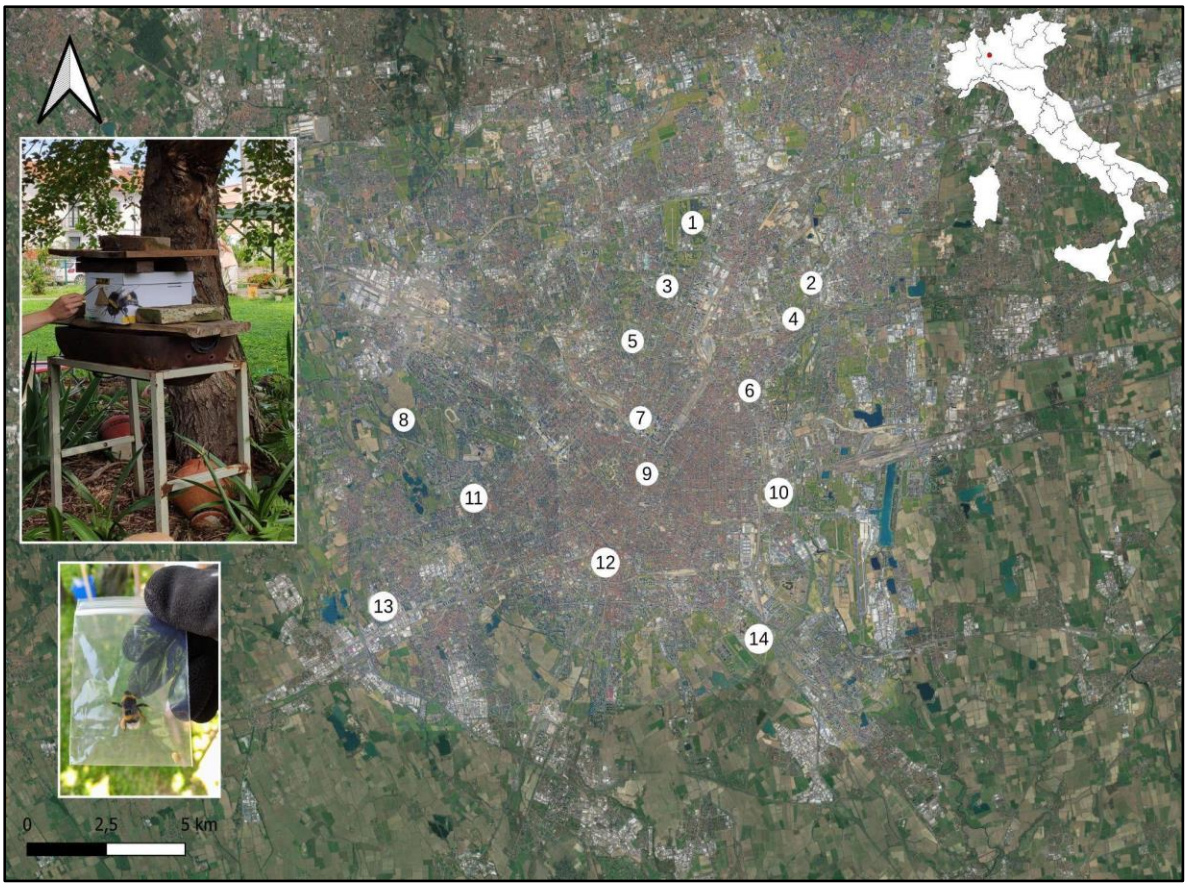

Figure 1. Map of the study area in central north Italy, showing the position of the experimental sites. The two boxes showed the setting of the colonies and a phase of the pollen sampling procedure.

Sampling of pollen loads and field data collection

Pollen loads were sampled from foragers just before entering their colonies after the foraging bouts. To collect pollen and avoid contamination, single workers were gently and individually placed in clean small plastic bags and the pollen pellets were detached from the corbiculae by gentle pressure on the pellets with tweezers. We sampled pollen from on average 28.25 ± 9.78 workers for each colony on each date, thus yielding a total of 3124 pellets weighing on average 19 mg (± 14) (dry weight). Samples were stored on ice on the field and then conserved at -80 °C until analysis.

To test for variations in the foraging behaviour of the colonies, the colonies in all sites were monitored using video cameras for three hours on a single day (SuperEye RJ0090-UK, OneThingCam^{TM}), a time comparable to a previous study (Biella et al. 2019). The number of workers entering and leaving the colony was later counted. We calculated the ratio between the egress and ingress of workers in intervals of 20 minutes as an index to estimate the foraging rate (Biella et al. 2019); for instance, a higher number of egresses compared to ingress per time units is a proxy of longer foraging bouts.

Phytochemical analysis

Individual pollen pellets sampled from the same colony were pooled to gain a total of about 4 replicates (mean = 3.7 ± 0.9) for each sampling date which corresponded to 7.78 ± 1.70 pollen pellets each. Aggregated pools were freeze-dried and ground into a fine powder using a TissueLyser (Qiagen, Germany). Samples were analysed for their protein, lipid, and carbohydrate content with the standard analytical protocols (Appendix S1, Text S1). Pollen was also analysed for its antioxidants content, total phenol content and flavonoid content as described in Appendix S1 (Text S1).

Pollen genetic identification

Pollen plant composition was assessed with a DNA metabarcoding approach. DNA extraction and sequencing were performed on the same pooled samples used for the phytochemical analysis, following standard protocols (Appendix S1, Text S2). Briefly, the ITS2 DNA region was targeted, and the library preparation and sequencing were conducted with Illumina MiSeq 600 V3 (2 × 300-bp paired-end sequencing). The obtained sequencing reads were processed with a standard bioinformatic pipeline to obtain ESVs (Exact Sequence Variants) to be assigned

taxonomically using a curated genetic reference database. Following the recommendation provided by Tommasi et al. 2021, the resulting plant identities in the samples were filtered with ROC curves based on sequencing reads distribution to gain ecologically realistic plant assemblies (See Appendix S1, Text S2 for all additional details). The number of plant species found in the pollen samples was used as an indicator of pollen richness.

Landscape fragmentation and local metrics

We used the regional land use cartography (DUSAF 6.0 https://www.dati.lombardia.it/Territorio/Dusaf-6-0-Uso-del-suolo-2018/7rae-fng6) to obtain data on "Green cover", by assigning to the original level and sub-level of land-use classification that defined polygons dominated by green patches (detailed information in Appendix S1 text S3). Using QGIS 3.10.11 we computed a buffer of 1 km radius around each site as this distance is above the mean foraging distance observed for bumblebees in urban landscape (Conflitti et al., 2022). Three fragmentation landscape metrics were calculated using the package *landscapemetrics* (Hesselbarth et al., 2019) in R ver 4.2.0 (R CoreTeam 2022). In detail, we quantified the percentage of green area cover (% Green Cover), the mean Euclidean nearest neighbour distance (ENN) and the contagion index (CI) as indexes of fragmentation.

The local assemblage of flowering plant species around the colonies was recorded by counting the number of species during random inspection walks replicated three times at each site; at least 100 m^2 of green areas were carefully investigated.

Statistical analyses

The effects of landscape fragmentation and of the number of flowering species on the nutritional profile of pollen were tested by using (Generalized) Linear Mixed Models

(LMMs/GLMMs) in R, with a binomial or beta distribution (based on the overdispersion parameters) distribution of the errors for variables of the percentages of protein, lipid, carbohydrate, antioxidant, phenol, and flavonoid content. A Gaussian distribution was used for the protein:lipid ratio (P:L ratio) and protein:carbohydrates ratio (P:C). The models included the fragmentation metrics (percentage of green cover, ENN, CI) and the number of flowering species as fixed terms and the identity of the site, colony nested within the site, and week of the sampling as random effects. Collinearity among the four covariates was evaluated through the variance inflation factors (GVIF). The eventual transformation of the predictor variables was evaluated through the AIC criterion (ΔAIC > 2, Zuur et al. 2009) and is reported in Table 1. The analyses were performed with *glmmTMB (*Brooks et al. 2023).

The relation between fragmentation metrics (cover, ENN, CI), the number of flowering species and the foraging rate was analysed by using Gamma distributed GLMMs with the identity of site and colony (nested within site) as random effects.

The data on colony weight in time relative to the initial weight were analysed by using Gamma distributed GLMMs with the ratio between the weight at a certain sampling replica and the initial weight as the response variable, the identity of site and colony (nested within the site) as random effects and the number of the sampling replica as the fixed effect.

Variations in pollen species richness in response to the fragmentation metrics (cover, ENN, CI) and to the number of flowering species recorded nearby the colony were evaluated through GLMMs with a Poisson distribution including the identity of site and colony (nested within site) and week of the sampling as the random effects. Furthermore, variations in time in the number of species foraged along the sampling season relative to the initial richness were evaluated by using Gamma distributed GLMMs with the ratio between the number of

species at a certain sampling date and the initial number as the response variable, the identity of site and colony (nested within site) as random effects and the number of the sampling replica as the fixed effect.

To test an association between pollen nutrient profile and plant composition, a NDMS ordination analysis of the plant composition per site was performed and subsequently correlated to the nutrient profiles. We analysed community count data using sequencing reads as proxies of pollen abundances and Bray-Curtis dissimilarity index, as recent studies support the correlation between ESVs count on pollen species to microscopic pollen grain counts (e.g. Keller et al. 2015); however, to avoid the influence of differential read abundances among plants, the same type of analysis was repeated also with presence/absence data (Appendix S1, Table S1). We used the function *metaMDS* from the package "*vegan"* in R (Oksanen et al. 2022). Directional cosines between each NMDS vector and each of the nutritional variables considered were tested and their significance was evaluated with the *envfit* function of the package "*vegan"*.

To test whether species composition varied across the different aspects of habitat fragmentation and to assess the association between the geographical origin of the plants in the pollen and landscape fragmentation we used the RQL and Fourth Corner analysis. Community count data (i.e., number of sequencing reads) were used as indicators of pollen abundance. Plant origin as native or exotic/invasive was assigned according to Biella et al. 2022 and Galasso et al. 2018. The analysis was performed with "ADE4" package in R (Dray & Dofour 2007). To test if species composition varied across different habitats the function *manyglm* was used. To assess if species abundance variation in response to fragmentation was related to their geographical origin the trait by environment interaction term was calculated

with the function *traitglm*. We set 999 permutations of sites and species values for testing significance.

Results

Pollen nutrition and colony features

The mean content of macronutrients of the pollen load dry weight were protein 14.72% (± 4.33%), lipid 4.67% (± 1.44%) and carbohydrates 30.48% (± 7.25%). The mean P:L ratio was 3.43 (± 1.38). Pollen nutritional content varied significantly in response to the amount and fragmentation of green areas in the investigated urban gradient matrix (Table 1). Specifically, pollen protein content showed a quadratic non-linear relationship with ENN, peaking at intermediate levels (Fig. 2a) and a significant increase in response to the higher number of flowering species (Fig. 2b). Lipids concentration increased quadratically with the amount of green cover with a peak around 30% of green cover (Fig. 2c). The P:L ratio decreased significantly in response to an increase in the ENN (Fig. 2d). Total polyphenols content was significantly higher when more flowering species were present (Fig. 2e). Significantly longer foraging bouts were only associated with a decrease in the amount of green cover at study sites (Fig. 2f, Table 1). Colony weight did not vary significantly during the sampling season with only a slight reduction at the end of the sampling season (Appendix S1, Table S2, Fig. S1).

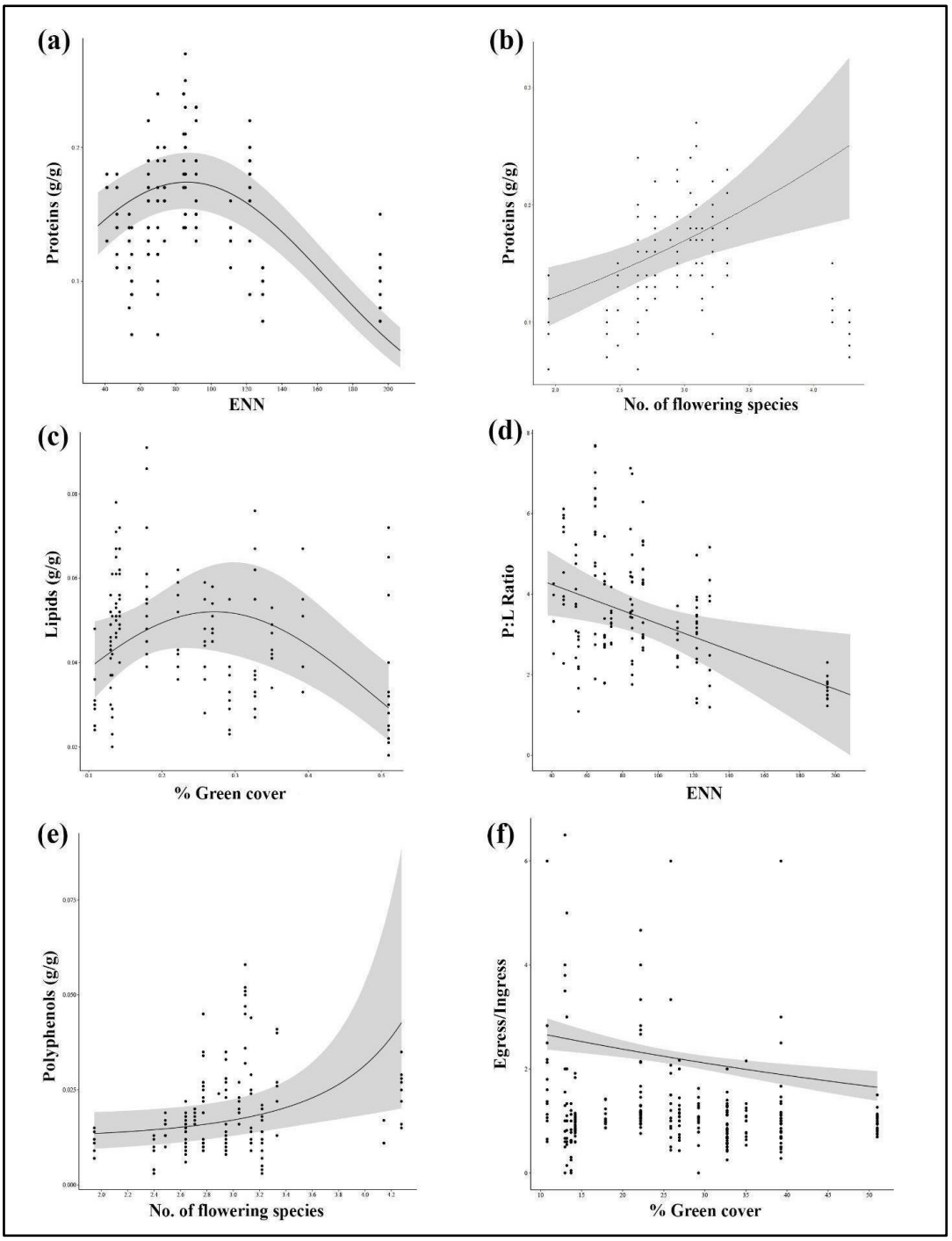

Figure 2. Graph showing the variation of pollen loads nutritional content and foraging rate in response to landscape metrics and richness of flowering species. (a) Variation of protein content in response to the ENN; (b) relation between protein content and the number of flowering species at the sampling sites; lipid concentration in relation to the amount of green cover is reported in (c) while the variation of protein to lipid ratio in (d). (e) shows the relation between the polyphenols concentration and the number of flowering species while in (f) is reported the relation of the variation in the foraging rate of the colony in response to the amount of green cover. The black lines and grey areas indicate the prediction of the model and its confidence intervals (α = 95%).

Table 1. Output of the regression analyses of the nutritional content of pollen and of the foraging rate as a function of green cover and fragmentation and the number of flowering species. The regression coefficient (βi), chi-square value (χ2), and p-value are reported.

Response variable	Model covariates	B_i	χ2	p-value
Proteins	% Green cover	-0.529	2.526	0.11
	ENN	1.019	13.975	**<0.001**
	I(ENN)^2	-1.412	15.237	**<0.001**
	CI	-0.051	2.415	0.12
	log(Number of flowering species)	0.017	6.55	**0.01**
Lipids	% Green cover	0.705	4.14	**0.02**
	I(% Green cover)^2	-0.792	5.571	**0.04**
	ENN	0.033	0.209	0.65
	CI	0.058	0.908	0.34
	log(Number of flowering species)	0.06	0.837	0.36
Carbohydrates	% Green cover	-0.05	0.408	0.52
	ENN	-0.022	0.1	0.75
	CI	-0.018	119	0.73
	log(Number of flowering species)	0.121	0.646	0.42
P:L ratio	% Green cover	0.075	0.11	0.74
	ENN	-0.644	4.756	**0.03**
	CI	0.279	1.839	0.17
	log(Number of flowering species)	0.088	0.0310	0.86
P:C ratio	% Green cover	-0.212	0.246	0.62
	ENN	-0.076	2.05	0.15
	CI	0.0381	0.813	0.37
	log(Number of flowering species)	-0.035	0.109	0.74
Antioxidants	% Green cover	-0.096	0.65	0.42
	ENN	0.09	0.931	0.33
	CI	0.069	0.019	0.89
	log(Number of flowering species)	0.195	0.643	0.42
Polyphenols	% Green cover	0.157	2.921	0.08
	ENN	-0.089	1.085	0.29
	CI	-0.037	0.372	0.54
	log(Number of flowering species)	0.562	7.613	**0.006**
Flavonoids	% Green cover	-0.092	0.874	0.35
	ENN	-0.03	0.099	0.75
	CI	-0.094	1.631	0.2
	log(Number of flowering species)	0.36	2.795	0.09
Egress/Ingress	% Green cover	-1.115	9.562	**0.002**
	ENN	-0.062	2.638	0.1
	CI	-0.029	0.751	0.39
	log(Number of flowering species)	-0.128	2.86	0.09

Pollen DNA metabarcoding

The transported pollen richness did not vary significantly in response to any of the three landscape metrics investigated neither to the richness of flowering species (Appendix S1, Table S3) nor along the sampling season (Appendix S1, Table S2, Figure S1). The RQL fourth corner analysis indicated that the plant community composition was significantly related to the amount of green cover but not to the ENN and CI indexes (Appendix S1, Table S4, Figure S2) while no significant correlation was found between plant geographical origin and landscape fragmentation by the Fourth Corner Analysis (Appendix S1, Table S5, Figure S6).

The NMDS analysis revealed that nearly all nutritional classes were significantly correlated with the specific composition of the plant communities (Appendix S1, Table S6). Higher protein content was correlated with communities dominated by species belonging mostly to the Fabaceae family (Fig. 3A), which were furthermore characterized by intermediate levels of lipids (Fig. 3B), carbohydrates (Fig. 3C) and phytochemicals (Fig. 3D, 3E, 3F).

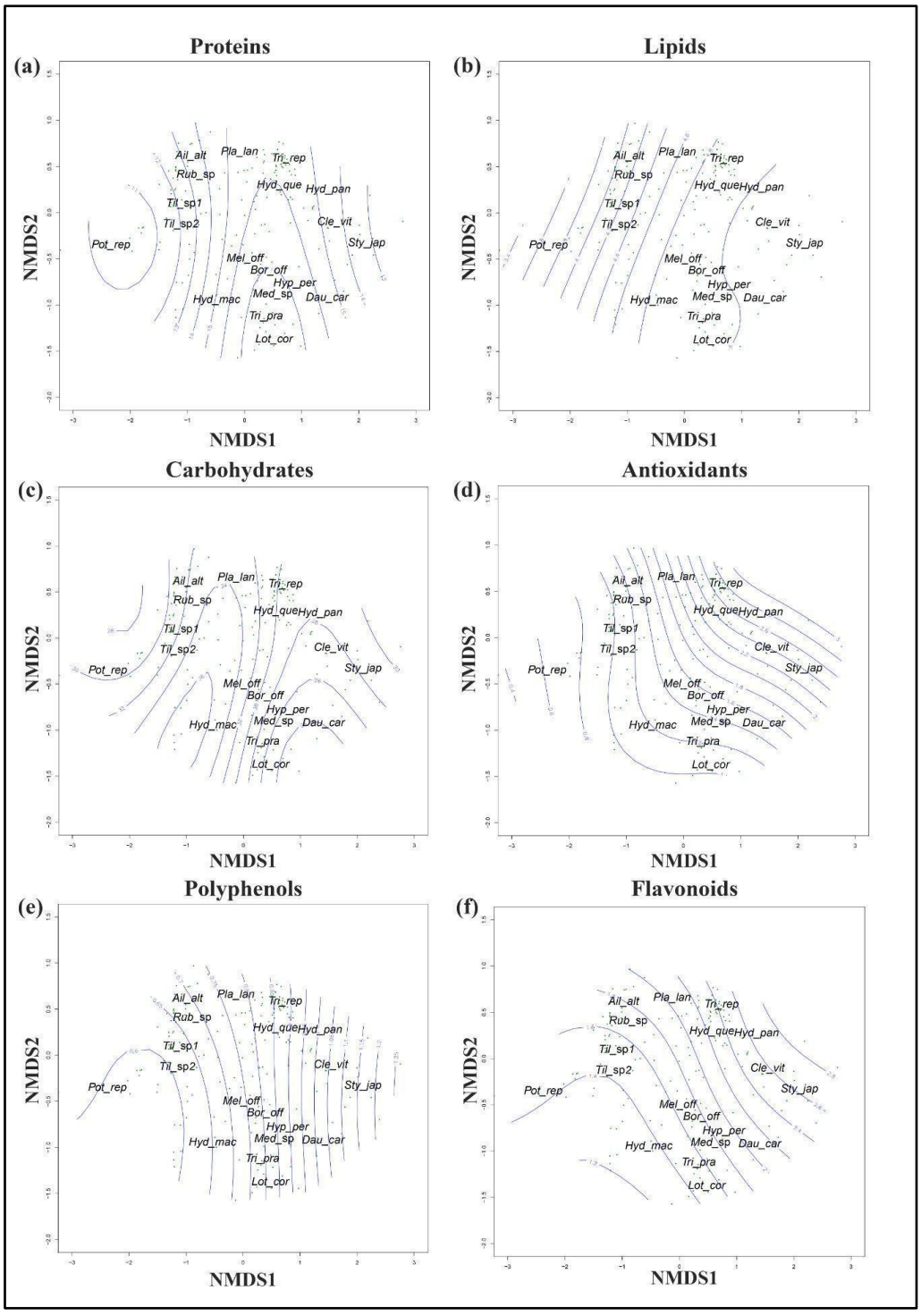

Figure 3. Surface non-metric multidimensional scaling (NMDS) ordinations of pollen plant communities are depicted in the figure. Black names indicate species centroids indicating samples dominated by these species (only the name of the most common species that covered more than 95 % of the total reads are reported). Blue contour lines indicate corresponding nutrient contents that correlated with ordinal axes, which are interpreted as how each species in the community (and the overall community composition) correlate with the nutritional contents the pollen transported. Full species name are reported in Appendix S1; Table S7.

Discussion

In this study, we investigated how habitat fragmentation could shape the foraging dynamics and nutritional intakes of a bumblebee species. By setting a field-based experiment using urban landscapes as an open-air laboratory where green patches of different size, isolation and floral availability are interspersed in often inhospitable concrete-dominated surfaces, we clarified how fragmentation could drive foraging for nest provisioning of a common pollinator. To address this topic, we looked at: diet nutritional quality, plant composition and foraging rate.

Our results underline the significance of habitat fragmentation and local floral diversity in the nutritional composition of *B. terrestris* pollen pellets in terms of protein, lipid, polyphenol, and protein:lipid ratio. These findings support our initial hypothesis that fragmentation plays a key role in decreasing the quality of resources collected by pollinators in a scenario where spatial constraints prevent accessing profitable resources. If compared with previous studies (e.g., Moerman et al. 2017; Vaudo et al. 2018), our results suggest a general impoverishment of diet quality. In contrast with a recent study by Vaudo et al., 2018 on *B. impatiens* where no nutritional content variations were found in the foraged pollen across green areas in agricultural (thus permeable) landscapes, our approach demonstrated that the urban matrix presents peculiar conditions that could impose more severe hindrance to the foraging activity of insect pollinators. This is clearly indicated by our results on the nutritional content of pollen pellets. Although some relationships between nutritional content and landscape features were linear, both protein and lipid contents displayed quadratic responses, with higher concentrations observed at intermediate levels of green cover and distances between green patches. Numerous studies have already emphasized how intermediate levels of urbanization,

for instance, those observed in suburban areas, can create favourable conditions for the thriving of pollinator populations (Banaszak-Cibicka & Żmihorski, 2020; Biella et al. 2022), and our results hint for a link with their diet quality. This result further strengthens previous observations that for the understanding of pollinator dynamics, it is important to account for green areas heterogeneity and for the variation in environmental features within different city realities (Ayers and Rehan, 2021).

The adverse impact of extreme levels of habitat fragmentation becomes more pronounced when examining the P:L ratio, which exhibits a linear decrease at increasing ENN. Previous research has indicated that bumblebees tend to prefer a pollen diet rich in protein (Roulston et al. 2000) and that they are able to actively select for pollen provisions with a higher P:L ratio under optimal conditions (Vaudo et al. 2018). Therefore, considering that we analysed pollen pellets destined to larvae feeding, our observations indicate a possible deterioration in bumblebee nutritional balance at sites with high fragmentation. It should also be acknowledged that the decrease in the P:L ratio could lead to developmental problems and to a decrease in insects' reproductive fitness (Vaudo et al. 2018; Manning et al. 2007).

Noteworthy, the local species richness of flowering plants was associated with the nutritional quality of the pollen collected, specifically in the protein and polyphenol contents. Polyphenols have been linked to numerous beneficial effects on bee health, including increased detoxification rates (Hýbl et al. 2021), improved memory retention (Riveros & Gronenberg, 2022), and mitigation of oxidative stress (Dordievski et al. 2023). Furthermore, experiments on *Apis mellifera* revealed a preference for sugar solutions containing polyphenols (Liu et al. 2006). In this context previous studies documented that local flower richness has a beneficial effect and correlates positively with local pollinator richness has been

documented (Ollerton 2017), and it is relevant also in urban context (Rajbhandari et al. 2023.). Our nutritional analysis showing responses to landscape fragmentation and to local flowering richness further supports the existence of a link between diversity/abundance trends and the nutritional aspects of the diets provisioned by these insects. This could also be mediated by the plant composition of the pollen collected to feed the larvae. The analyses confirmed a tight association between pollen plant composition, the nutritional profiles, and landscape, and no variation in pollen species richness along the fragmentation gradient. This result supports the hypothesis that fragmentation constrains the access to profitable resources; otherwise, if foragers were highly adaptive, then alternative local resources of high quality or a higher species richness would have been collected even in fragmented landscapes and no plant composition-nutrient association would be found (Barraquand & Benhamou 2008, Biella et al. 2019). Furthermore, the analyses suggest that while the overall number of species foraged remained consistent, the composition of the diet represents the main driver of the changes in the observed variation of pollen nutrition. In agreement with this, the fourth corner analysis confirmed that a landscape feature as the green area size shapes the specific composition of the collected pollen communities. However, this landscape influence was not reflected by the prevalence of native or exotic species in the pellets, indicating that plant species nutritional idiosyncrasies, other than plant origin, play a role in resource selection of the foraged pollen. This latter finding contrasts with previous studies that recorded the role of ornamental or exotic plants in pollen of an urbanization gradient and the role of garden plants for feeding pollinators in an urban context (Biella et al. 2022; Staab et al. 2020; Tew et al. 2021). This is probably because our study was entirely set in an urban context of quite similar habitats (i.e., urban parks) rather than comparing different urban ones. Overall, these findings highlight the complex relationships between landscape characteristics, plant

community composition, and the nutritional resources available to bumblebees, leaving open the question of possible behavioural adjustments by foragers.

To answer this, we recorded workers leaving and returning the nests and found that the foraging effort by the colony, measured as the ratio between leaving and returning by time unit, showed a significant negative relationship with the amount of green cover surrounding the study sites. In other words, increasing green cover leads to shorter duration of the foraging trips. Indeed, as the foraging effort reflects the colony needs and the spatial accessibility of resources (Jha & Kremen 2012; Pernal & Currie 2001), longer trips were performed in landscapes with less green areas, as expected. In areas with less cover where patches are less profitable, a forager would forage longer, for instance for maximizing quantity over quality or due to high searching time for good resources (Weterings et al. 2018; Jha & Kremen 2012). Conversely, with a high green cover and according to the optimal foraging theory, pollinators will forage close to their nesting site in landscapes with evenly distributed resource patches to reduce searching costs and maximize foraging efficiency (Heinrich 1979). This difference in fragmentation causing differences in foraging efficiency could affect the fitness of the colonies as they are strongly influenced by foraging behavioural traits, including foraging trip duration (Westphal et al. 2006). Furthermore, changes in the foraging pattern as the one we observed in this study can impact how pollen is distributed across the landscape and ultimately results in an impairment of the pollination service (Kremen et al. 2007).

Conclusions

In conclusion, this study clarified the role of fragmentation in shaping foraging dynamics when it comes to collecting resources for nest provision in a common bumblebee as *B.*

terrestris. It also raises several warnings on the limited capacity of the urban nutritional landscape to sustain social pollinator populations. It is still to be understood if reinforcement programmes aimed at increasing flower diversity in cities go along with the nutritional needs of pollinators and if such interventions will reverse the negative trends observed here with high urban fragmentation levels. Landscape-scale conservation efforts should prioritize the preservation and restoration of green habitats with suitable floral resources to support the foraging behaviour and population persistence of bumblebees, especially in contexts threatened by high human-driven fragmentation. Alongside the management and creation of these conducive habitat, mitigation actions should also be directed towards enhancing the connectivity among green patches to increase accessibility to food resources for insect pollinators. This multifaceted approach, integrating habitat provision, management, and connectivity, will play a key role in safeguarding pollinators populations and their vital ecological contributions within urban environments.

FUNDER

Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of the 18 December 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU.

Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP, H43C22000530001 Project title “National Biodiversity Future Center - NBFC”.

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APPENDIX S1

Text S1 - Methods for pollen nutritional analysis

Extraction and quantification of protein content

To analyze the protein concentration of pollen loads we performed the Bradford assay. For each sample, approximately 1 mg of pollen was weighed in triplicate and added to a 300 µL 0.1 M NaOH solution. To allow the mechanical lysis of the pollen wall, the samples were subjected to 3 cycles of direct sonication lasting 10 seconds each by an ultrasound probe sonicator (Branson, USA). At the end of the process, 1.2 mL 0.1 M NaOH was added, and the samples were maintained at 4°C for 24 h to allow the chemical lysis and then centrifuged at 14,000 x g for 5 minutes. The extracts were tested performing the Bradford assay using the Coomassie brilliant blue dyer (ThermoScientific, USA) and the bovine serum albumin or BSA (Merck, Germany) as analytical standard (range 0-1500 µg/mL). Samples absorbance was measured at a wavelength of 595 nm against the blank. The entire process was performed by keeping the samples on ice to avoid possible protein degradation phenomena.

Extraction and quantification of lipid and carbohydrates

For the extraction of lipids and carbohydrates, a volume of 200 µL of Na_2SO_4 2% w/v was added to 1 mg of pollen (in triplicate for each sample) and then the mechanical

lysis was performed by probe ultrasonication as above. Then samples were transferred to a glass tube and 1.6 mL of a 1:1 v/v $CHCl_3$/MeOH solution was added. The tubes were vortexed and centrifuged for 5 min at 3,200 x g. The supernatant was transferred to a clean glass tube, then 600 µL of ultrapure Milli-Q H_2O was added and the samples were centrifuged at 3,200 x g for 5 minutes. This process led to the formation of two clearly different phases: the upper one composed of MeOH and H_2O containing carbohydrates and polar molecules, and the underlying one made of $CHCl_3$, containing lipids. The two phases were separated and the whole process was repeated twice to maximize the extraction efficiency of the analytes of interest.

For the quantification of lipids, we conducted the Vanillin assay using soybean vegetable oil as standard (range 2.5-400µg). The samples were soaked at 60°C until complete evaporation of $CHCl_3$. Hereafter, 200 µL H_2SO_4 98% v/v solution was added, and the temperature was raised up to 100°C. After 10 min, 4.8 mL of vanillin (1.2 mg/mL in 68% v/v H_3PO_4) was added. Samples were read against the blank at the wavelength of 490 nm.

For the quantification of sugars, a solution of 1.4 mg/mL of anthrone was prepared in H_2SO_4 72% v/v and glucose was used as an analytical standard (0 - 200 µg/mL). Meanwhile, the samples were heated at 45°C using a Speedvac (Eppendorf Concentrator Plus, Germany) up to dryness. Then, the dry extract was resuspended

in 400 µL of a solution made of anthrone/H_2O (3:1 v/v) and subsequently vortexed to facilitate dissolution. Further dilutions were done by using the anthrone solution. Then, 1.6 mL of anthrone solution was added and the samples were heated at 100°C for 17 min in a thermomixer (Falc, Italy). After allowing the samples to cool down, the absorbance of samples was read against a blank at a wavelength of 625 nm.

Extraction and quantification of secondary compounds content

To extract the secondary metabolites from pollen loads, a 1 mg pollen sample was extracted in 1 mL of MeOH 70% v/v for two extraction cycles lasting 10 minutes each by using a bath sonicator (Argolab, Italy) at a frequency of 37 Hz and at a fixed temperature of 30°C. At the end of each extraction, the samples were centrifuged at 14,000 x g for 3 minutes and the supernatant was recovered. The extraction was performed three times.

To quantify the antioxidant activity of the extracts the DPPH (2,2-DiPhenyl-1-picrylhydrazyl) assay was performed using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as analytical standard (0 - 500 µmol/L). In a quartz cell, 50 µL of the sample was incubated with 950 µL of 0.1 mM DPPH solution. A triplicate reading was made for each sample. After 30 minutes of incubation at room temperature in the dark, the samples were read at a wavelength of 515 nm against the blank.

The quantification of the total phenol content of the extracts was performed by the Folin-Ciocalteau assay using gallic acid (Merck, Germany) as analytical standard (range 0 - 100 µg/mL). Then in a quartz cell, the following solvents/solutions were added: 400 µL H_2O milli-Q, 80 µL sample, 40 µL Folin-Ciocalteu reagent (Merck, Germany) and 480 µL Na_2CO_3 10.75% w/v. The whole volume was mixed and incubated for 30 minutes in the dark at room temperature. The samples were read at a wavelength of 760 nm against the blank.

For the quantification of the total flavonoid content, the $AlCl_3$ assay was carried out using quercetin (Merck, Germany) as the analytical standard (0- 50 µg/mL). Subsequently, 400 µL of H_2O milliQ, 340 µL of the sample, and 30 µL of $NaNO_2$ 5% w/v were added to a quartz cell. The whole volume was mixed and left to incubate for 5 minutes. Hence, 30 µL $AlCl_3$ 10% w/v was added, followed by further 6 minutes of incubation. Finally, 200 µL NaOH 1 M were added and the solution was mixed thoroughly before measuring the absorbance at the wavelength of 415 nm against the blank.

Text S2 - Methods for pollen DNA metabarcoding

Pollen samples were frozen in liquid nitrogen and ground using a Tissue Lyser® II (Qiagen©, Hilden, Germany). Their DNA was extracted in a laminar-flow laboratory cabinet with the Qiagen© DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The final elution volume was 50 µl and

negative control was produced during each DNA extraction phase. The internal transcribed spacer 2 (ITS2) amplification was conducted using S2F and S3R primers tailed with Illumina overhang sequence adapters (Chen et al., 2010). For the library preparation and the sequencing an Illumina Miseq 600 V3 (2 × 300-bp paired-end sequencing) technology was performed (San Raffaele Scientific Institute, Milan, Italy). The following bioinformatic pipeline involved QIIME2 2021.4 (Bolyen et al., 2019). After trimming primers, DADA2 algorithm was used to denoise and demultiplex sequences by applying a quality filter (expected error in forward 2, in reverse 4, quality score 1) and kept each strand sequence in the range of 275-245bp. This procedure was performed independently for each sequencing run. The obtained ESVs and representative sequences were taxonomically assigned using VSEARCH alignment algorithm and the MetaCurator reference dataset (Richardson et al. 2020), and consensus taxonomy for each sequence was obtained. In addition, the output of the taxonomic identification was further validated by visually inspecting the neighbor-joining tree of all ESVs and for the unidentified ESVs we attempted to manually identify them using Basic Local Alignment Search Tool (BLAST) against the public NCBI Genbank based on the highest identity score and only if above 98%.

The obtained ESVs table with assigned taxonomy was subjected to ecological filtering based on ROC curves as in Biella et al. 2019, which finds cut-off thresholds

accounting for the distribution of reads among molecular features. We associated a variable coded as “negative” or “positive” to each taxon of a sample: “negative” if reads were 0, otherwise, “positive”; We fitted a Generalized Linear Regression with an overdispersed Poisson distribution (quasi-Poisson) for each sample, and with the *pROC* package in R (Robin et al. 2011), the ROC curves were built between the estimated reads and the actual “positives”/“negatives”; based on the Youden’s J statistic, an optimal cut-off threshold was obtained with the function *coord* in the same package (Youden 1970) and then set to zero all features with reads below the threshold.

Sequencing results

A total of 33,582,616 raw reads were produced after High Throughput Sequencing of the pollen ITS2, with an average read length of 445 bp (± 27 bp). After the filtering, a total of 11,110,996 reads were obtained and the inferred ESVs were classified into 25 families, 42 genera and 50 species with each sample containing an average of 3.1 species (± 1.4), 3.7 genera (± 1.2) and 2.5 families (± 1.0).

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Text S3 - Methods for land-use analysis

Landscape composition was characterized in a buffer area of 1 km around each sampling site and based on the DUSAF 6.0 land-use cartography. (2018 - DUSAF 6.0; https://www.dati.lombardia.it/Territorio/Dusaf-6-0-Uso-del-suolo-2018/7rae-fng6 accessed 26/06/2021)

This map is available at a scale of 1: 10.000 with a minimum linear dimension of polygons of 20 m and was developed from AGEA orthophotos and SPOT 6/7 satellite images.

We grouped similar land uses into broader types mostly based on the Level 1 classification, and calculated the percentage of green cover by summing the area of all the polygons that were characterized by a dominance of green patches.

In detail: DUSAF land use level type "11231", “1411”, “1412”, “2115”, “2241”, “2242”, “2311”, “2312”, “31111”, “31112”, “31121”, “3113”, “314”, “3222”, “3223”, “3241”, “3242”, “411” were categorized as “Green area”.

(Full explanation of codes is available at https://www.cartografia.regione.lombardia.it//metadata/Dusaf/doc/Legenda_DUSAF_2018_6_0.pdf)

Table S1. Results from plant composition and nutritional content -NMDS output with the correlations between plant community data (presence/absence) and nutritional content of the pollen foraged.

	NMDS1	NMDS2	r^2 Correlation	*p*- value
Proteins (g/g)	0.93133	0. 36417	0.0892	**0.01**
Lipids (g/g)	0.94794	-0. 31844	0.0688	**0.01**
Carbohydrates (g/g)	-0.53506	-0.84482	0.0717	**0.01**
P:L ratio	-0.35743	0.93394	0.0063	0.56
P:C ratio	0.66264	0.74894	0.1539	**0.01**
Antioxidants (g/g)	0.56204	-0.82711	0.2645	**0.01**
Polyphenols (g/g)	0.9955	0.09471	0.1774	**0.01**
Flavonoids (g/g)	0.85738	-0.51468	0.1275	**0.01**

Table S2. Results from time replicates in colony weight and pollen species richness - Output of Tukey's post-hoc analysis, the regression coefficient (βi) and p-value are reported.

Response variable	Model covariates	Comparison	B_i	*p* -value
Weight / Initial Weight	Replica	3 - 2 == 0	-0.003	0.993
		4 - 2 == 0	-0.090	**0.002**
		4 - 3 == 0	-0.087	**0.003**
Pollen richness / Initial Pollen Richness	Replica	3 - 2 == 0	-0.044	0.887
		4 - 2 == 0	0.038	0.952
		4 - 3 == 0	0.082	0.780

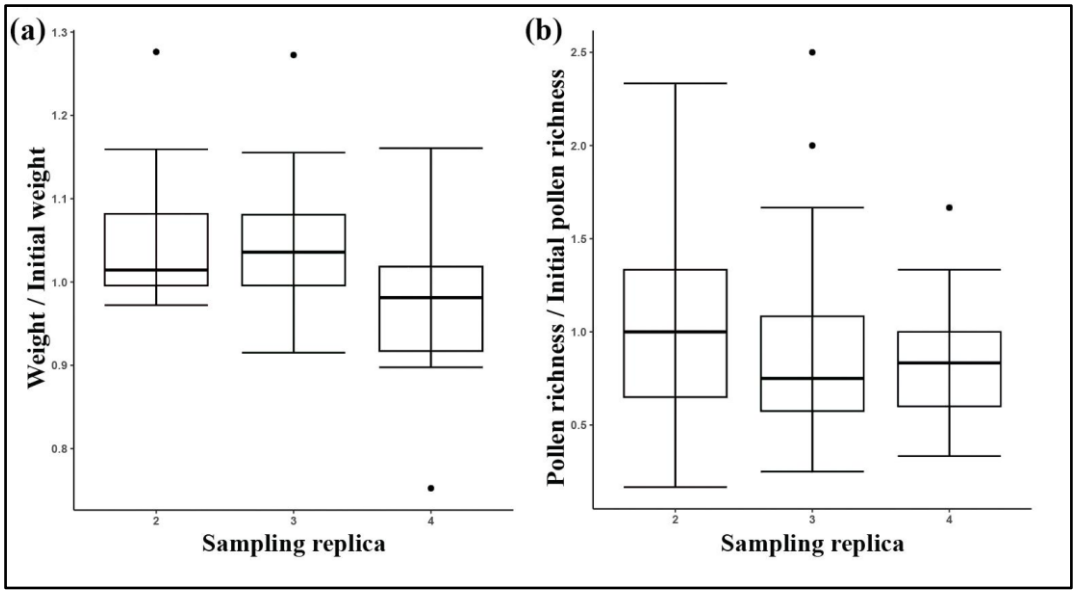

Fig. S1. Colony weight and pollen species richness across time replicates – Boxplot showing the (a) relative weight and (b) relative pollen richness variation recorded along the sampling season.

Table S3. Results of Pollen species richness in response to land-use - Output of the regression analysis of the pollen species richness as a function of the three landscape metrics and of the number of flowering species recorded at site. The regression coefficient (βi), chi-square value (χ2), and p-value are reported.

Response variable	Model covariates	B_i	$\chi 2$	p-value
Pollen richness	% Green cover	-0.019	-0.252	0.80
	ENN	-0.088	-0.95	0.34
	CI	0.094	1.434	0.15
	log(Number of flowering species)	-0.099	-0.533	0.59

Table S4. Results from RQL analysis between plant species composition and landscape fragmentation indices - Output of the analysis of deviance to test for a significant effect of landscape metrics on plant community data. Residual degrees of freedom, differences in degrees of freedom, Deviance and the p-value are reported.

	Res.Df	Df. Diff	Dev	*p*-value
Intercept	13			
% Green Cover	12	1	74.96	**0.04**
ENN	11	1	48.23	0.06
CI	10	1	41.78	0.27

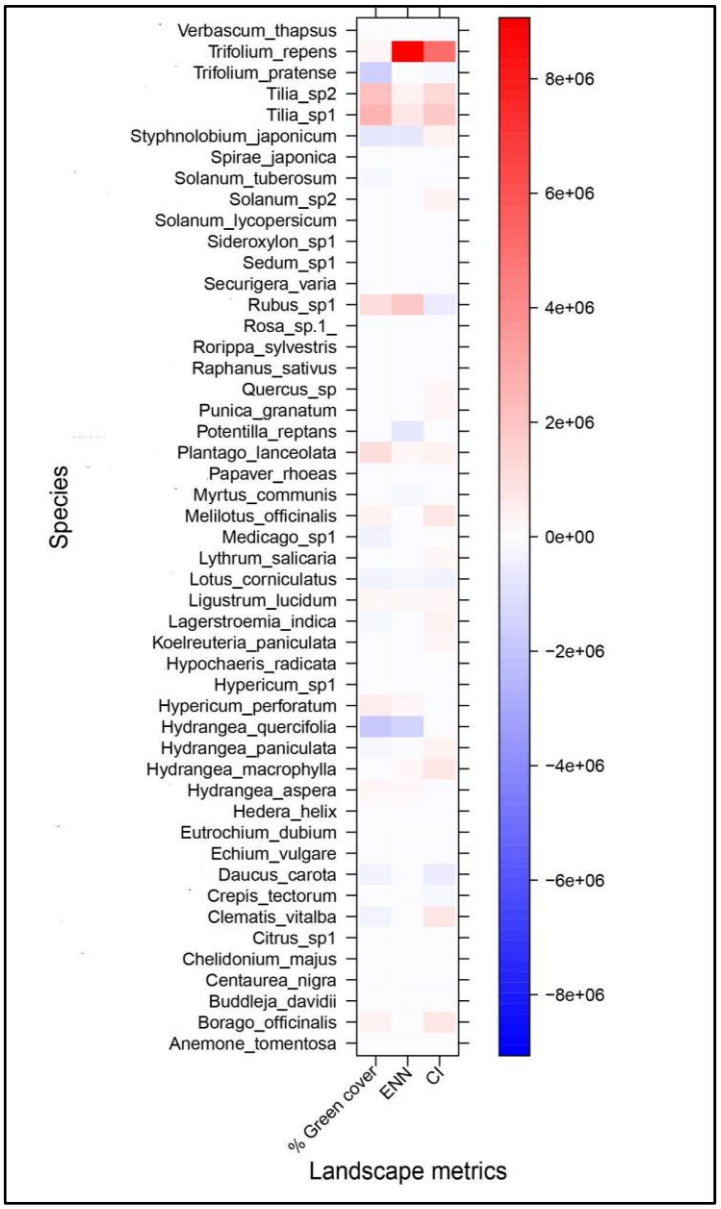

Figure S2. Association between plant species composition and landscape fragmentation indices – Heatmap representing species abundance in relation to landscape metrics. Positive correlations are shown in red and negative correlations are shown in blue. The fourth coefficients indicate the amount by which a standard deviation unit change in the environmental variable changes the slope of the relationship between abundance data and a given landscape metrics.

Table S5. Results from Fourth Corner analysis on plant origin and landscape fragmentation – Output of the analysis of deviance testing for a significant effect of trait by environment interaction term. Residual degrees of freedom, differences in degrees of freedom, Deviance and the p-value are reported.

	Res.Df	Df. Diff	Dev	*p*-value
Main effects only	644			
environment x plant origin (fourth corner)	638	6	23.85	0.174

Table S6. NMDS output with the correlations between plant community data and nutritional content of the pollen foraged.

	NMDS1	NMDS2	r^2 Correlation	*p*-value
Proteins (g/g)	0.74	0.67	0.11	**0.01**
Lipids (g/g)	0.99	0.79	0.08	**0.01**
Carbohydrates (g/g)	-0.67	-0.74	0.05	**0.01**
P:L ratio	-0.27	0.96	0.02	0.22
P:C ratio	0.62	0.78	0.17	**0.01**
Antioxidants (g/g)	0.66	-0.75	0.22	**0.01**
Polyphenols (g/g)	0.92	0.38	0.16	**0.01**
Flavonoids (g/g)	0.92	-0.4	0.11	**0.01**

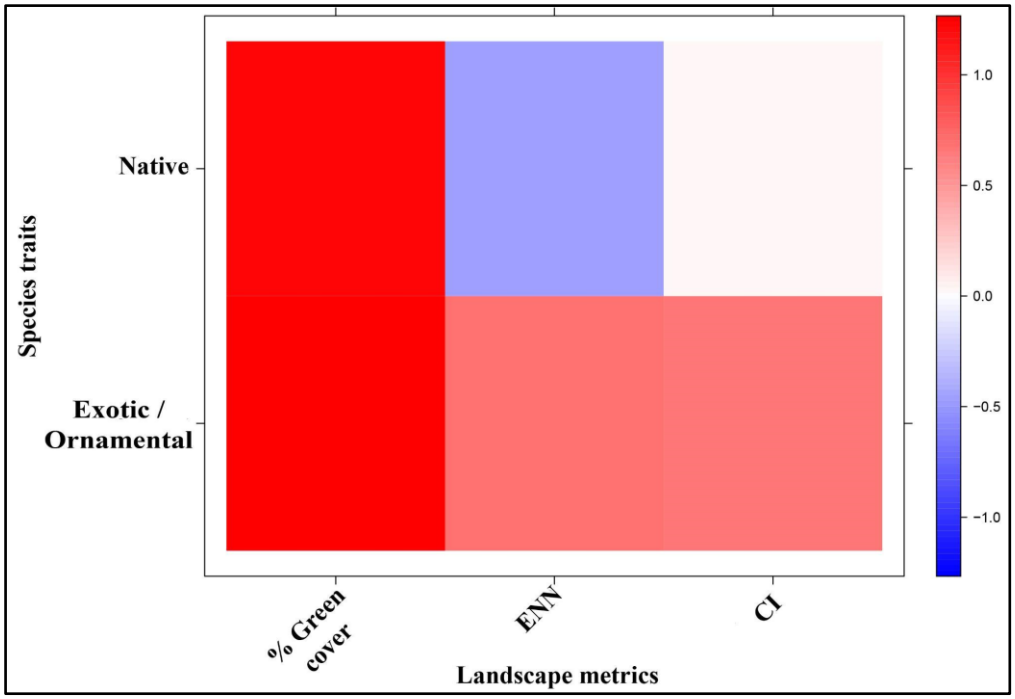

Figure S3. Relationship between plant origin and landscape fragmentation- Output from the fourth corner analysis showing correlations between landscape metrics and plants geographical origin. Positive correlations are shown in red negative correlations are shown in blue. Larger numbers show stronger correlations.

Table S7. – Abbreviation codes for plant species name - Table that report the full name of the species that are indicated in the abbreviated form in Figure 3 in the main text.

Abbreviated name	Full name
Ail alt	*Ailanthus altissima*
Ane tom	*Anemone tomentosa*
Bor off	*Borago officinalis*
Bud dav	*Buddleja davidii*
Cen nig	*Centaurea nigra*
Che maj	*Chelidonium majus*
Cit sp	*Citrus* sp*1*
Cle vit	*Clematis vitalba*
Cre tec	*Crepis tectorum*
Dau car	*Daucus carota*
Ech vul	*Echium vulgare*
Eut dub	*Eutrochium dubium*
Hed hel	*Hedera helix*
Hyd asp	*Hydrangea aspera*
Hyd mac	*Hydrangea macrophylla*
Hyd pan	*Hydrangea paniculata*
Hyd que	*Hydrangea quercifolia*
Hyp per	*Hypericum perforatum*
Hyp sp	*Hypericum* sp*1*
Hyp rad	*Hypochaeris radicata*
Koe pan	*Koelreuteria paniculata*
Lag ind	*Lagerstroemia indica*
Lig luc	*Ligustrum lucidum*
Lot cor	*Lotus corniculatus*
Lyt sal	*Lythrum salicaria*
Med sp	*Medicago* sp*1*
Mel off	*Melilotus officinalis*
Myr com	*Myrtus communis*
Pap rho	*Papaver rhoeas*
Pla lan	*Plantago lanceolata*
Pot rep	*Potentilla reptans*
Pun gra	*Punica granatum*
Que sp	*Quercus* sp
Rap sat	*Raphanus sativus*
Ror syl	*Rorippa sylvestris*
Ros sp	*Rosa* sp.1
Rub sp	*Rubus* sp1
Sec var	*Securigera varia*
Sed sp	*Sedum* sp1
Sid sp	*Sideroxylon* sp*1*
Sol lyc	*Solanum lycopersicum*
Sol sp	*Solanum* sp*2*
Sol tub	*Solanum tuberosum*
Spi jap	*Spirae japonica*
Sty jap	*Styphnolobium japonicum*
Til sp*1*	*Tilia* sp*1*
Til sp*2*	*Tilia* sp*2*
Tri pra	*Trifolium pratense*
Tri rep	*Trifolium repens*
Ver tha	*Verbascum thapsus*

Chapter III

Novel insights into the role of pollination in shaping fruits and seeds quality and chemistry

Case Study V. Pioltelli, E., Biella, P., Sala D., Guzzetti, L., Labra M., Galimberti A. (2023). Pollination on the table: insights into the role of insect pollination in shaping fruit and seed chemistry and commercial value

Status: Manuscript in preparation

The manuscript presented here is a first draft.

Pollination on the table: insights into the role of insect pollination in shaping fruit and seed chemistry and commercial value

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Abstract

The relevance of insect pollination in ensuring food security is widely recognized. While previous studies have underscored the role of pollinators in enhancing crop yield and commercial value, their potential influence on the chemical composition of crops, especially concerning secondary metabolites with significant implications for human nutrition, has been largely overlooked. In this study, we set an experimental trial with two species, *Fragaria vesca* L. and *Vigna unguiculata* L. Walp. Flowers from these species were exposed to three pollination mechanisms: i) forced self-pollination; ii) hand pollination and iii) open pollination. The strawberries and cowpea seeds produced were analyzed to assess commercial quality and chemistry by an untargeted metabolomic approach based on LC-MS/MS. Results showed that both species positively benefited from insect mediated pollination in terms of marketability parameters. Remarkably, beyond macroscopic features, the phytochemical composition was influenced by the pollination treatment. open pollinated strawberries exhibited a higher concentration of compounds with nutraceutical interest, such as anthocyanins, ellagic acid derivatives, and numerous flavonoids. In contrast, self pollinated strawberries and cowpea seeds displayed a higher occurrence of anti-nutritional compounds, such as tannins and saponins. These findings highlight the role of insect pollination not only in shaping the physical characteristics of fruits and seeds, but

also in determining their chemistry. This observation carries profound implications for both ecological dynamics and dietary considerations, underscoring the intricate interplay between pollinators, plants, and human nutrition.

Introduction

Among the multiple challenges posed by the exponential growth of the human population, ensuring access to an adequate quantity and quality of food emerges as a priority. The attention to this issue finds tangible expression in the Sustainable Development Goals (SDGs) 2 and 3 that are part of the Agenda 2030 for sustainable development adopted by the UN (United Nations-UN, 2015). Goal 2 aims to end hunger, achieve food security, improve nutrition, and promote sustainable agriculture while Goal 3 aims to ensure healthy lives and promote well-being for all at all ages. As many health issues, especially non-communicable diseases, are related to an insufficient or inadequate assumption of nutrients (Willet et al., 2019;), the interconnectedness between these two objectives is evident. Furthermore, the two goals share a strong dependency on the provision of ecosystem services and the conservation of functional biodiversity mediating them. In this context, the pollination service and pollinators play a crucial part. In fact, pollinators are more than just facilitators of flowering plant reproduction as they

also play a key ecological and socio-economic role at the global scale. The undeniable connection between pollination and human well-being is underscored by its incontrovertible economic value, primarily derived from its role in agricultural yield and food security (IPBES 2016; Smith et al., 2015).

Despite pollinator-dependent crops accounting for only 2.5 % of the global calories production (Ghazoul 2005; Richard 2001), they play a disproportionate role in dietary nutrient supply. Considering micronutrients, animal pollination contributes to providing up to 20 % of vitamin C, 41% of vitamin A, and 7% of folate (Smith et al. 2015), globally. Insufficient intake of key foods affected by pollinator species is indeed linked to an increased risk for non-communicable diseases (e.g., diabetes, cancer, and cardiovascular diseases) (Lim et al., 2012). Surprisingly, although the positive relations between animal pollination and crop yield/commercial grade (i.e., weight, shape, fruit color, firmness, and shelf life) have already been demonstrated (Garibaldi et al., 2013; Klatt et al., 2014), the influence of insect pollination on the nutritional and nutraceutical features of fruits and seeds have been largely unknown so far (Wietzcke et al., 2018). Only a few studies have hypothesized the influence of insect pollination on phytohormonal-driven changes associated with fruit ripening and taste such as an increment of sugar and acid concentrations in strawberries (Wietzcke et al., 2018; Rosianski et al., 2016), an increase of essential nutrients in seeds (e.g., vitamin E and unsaturated fatty acids in sunflower oil and

almonds [Silva et al., 2018; Brittain et al., 2014]) or the mineral content in commercial apples (Samnegård et al., 2019). All these processes strongly suggest that pollination mechanisms possess the capability to initiate or shape a wide array of plant metabolic pathways that ultimately can determine and impact the chemical composition of food. Despite this first evidence of the contribution of pollination to multiple facets of crop quality, a recent global meta-analysis by Gazzea et al. 2023 indicated that animal pollination service strongly improved multiple organoleptic and commercially important traits of fruits and vegetables, while it contributed to a lesser extent to food nutritional content.

Overall, a more precise investigation of the relationship between insect-mediated pollination and the nutritional features of agronomic products has the potential to pave the way for novel policy strategies aimed at promoting biodiversity conservation, facilitating the ecological transition of productive systems, and enhancing human well-being. These objectives align with the priorities delineated in the National Recovery and Resilience Plan (PNRR) and the directives of the EU Green Deal (EGD, European Commision, 2019) and of the recently proposed Nature Restoration Law (European Commission, 2022).

The core objective of this study is to investigate whether flower visitation by pollinator insects engenders modification in the characteristics of two crops relevant for food purposes and characterized by different features at the

morphological and phylogenetic level (i.e., *Fragaria vesca* L. and *Vigna unguiculata* Walp.).

Specifically, we looked at the alterations in the nutrient composition, with a particular focus on secondary metabolites potentially endowed with nutraceutical properties that, to date, no study has investigated yet. Additionally, the commercial quality and physiological parameters (e.g., seed germination rate) were also investigated. This comprehensive analysis seeks to provide an all-encompassing description of the effects of insect-mediated pollination on the nutritional value of food on the table.

Materials and Methods

Experimental set-up

A total number of 120 seedlings of *F. vesca* were obtained from Valitutto s.r.l (Sicignano degli Alburni, Italy) and 100 seeds of *V. unguiculata (accession number* TVU11733*)* were obtained from IITA (International Institute of Tropical Agriculture, Ibadan, Nigeria).

The *F. vesca* plants were cultivated in pots within an experimental open greenhouse and were divided into four distinct plots, each comprising 30 plants. Strawberry plants were grown in 9 L pots filled with a peat: pumice substrate (Hochmoor–Terflor, Capriolo, Brescia, Italy) and fertigated with a nutrient solution (Ferti 3,

Planta-Düngemittel, Regenstauf, Germany) every two weeks. Each plant was watered twice a day with 600 mL of tap water. The seeds of *V. unguiculata* were planted in an experimental open field with the same disposition described in Pioltelli et al., 2023a. The plants were grown in collaboration with the CREA (Council for Agricultural Research and Agricultural Economics Analysis) of Sanremo, Italy and two cultivation seasons for both species (years 2021 and 2022) were carried out.

Pollination treatments

Both F. vesca and V. unguiculata flowers underwent identical pollination treatments. Specifically, flower buds were subjected to one of three following conditions: i) in the self-pollination (SP) treatment, flowers were covered by using Osmolux bags (purchased from Pantek, France) to prevent pollination events different from autogamy, ii) the hand pollination (HP) was characterized by the application on the stigmas of selected flowers of pollen originating from a different individual by using a soft brush, and iii) the open pollination treatment (OP) were floral units were let free to be visited by pollinator insects, such as bees, flies, and butterflies. To obtain enough pollen required to enable the HP treatment, pollen was collected by means of the E–PoSa portable vacuum as described in Pioltelli et al., 2023b. The choice of the Osmolux bags for the SP treatment was based on their characteristics, which include semi-permeability for water and steam, ensuring

minimal differences in the microclimate between bagged and unbagged flowers, and high visible light transparency (Wietzke et al., 2018). The bags were removed directly at the beginning of the fruit set (i.e., after the fall of petals and the beginning of fruit swelling). Strawberry fruits were harvested at full maturity, while cowpea pods were collected at full fruit dehiscence. This was made to avoid any difference related to the ripening stage. Concerning *F. vesca*, we will define as "fruit" the combination of achenes and receptacles. Once collected, the fruits were analyzed for their morphological traits and then stored at -20°C prior to the laboratory analyses.

Morphometric analyses and quality assessment

Wild strawberry. morphology and commercial features estimate

The horizontal diameter, length, and weight of the treated strawberries were measured immediately after harvesting to avoid influence on post-harvest quality, owing to water loss and metabolic alterations. The fruit color was measured with a portable colorimeter (CR - 410 Chromameter, The Netherlands) at two opposite sides of the fruits in the L * a * b color space, and the a-value, which indicates the green-red composition, was recorded similarly to what reported in Wietzcke et al., 2018.

Total soluble solids (TSS), titratable acids (TA), pH values, and the number of fertilized achenes were measured in a subset of ripe fruits per treatment (for a total number of 20 strawberries per treatment). The fruits were homogenized with a tube mill (IKA, Germany) and subsequently centrifuged twice at 7000 rpm for 15 minutes. The supernatant was separated and used to determine the pH-value, TSS, and TA. Specifically, for the estimate of TSS a hand-held refractometer (Krüss, Germany) was used. The pH-value was obtained through a pH-meter (HANNA, USA) while the TA was quantified following the protocol proposed by Caner et al, 2008 and Wietzcke et al, 2018. Finally, the sugar-acid-ratio was calculated by dividing the TSS by the TA. Fertilized achenes were separated from unfertilized ones thanks to their different sedimentation abilities in water and were manually counted.

Cowpea seeds dimensions, germination, and primary metabolism

Pods length and weight were measured post-harvest. The total number of seeds per pod was recorded and the mean weight of seeds was measured. Also, the proportion of aborted seeds per pod was calculated.

For each pollination treatment, 100 seeds were tested for their germination rate. The seeds were disposed in five different petri dishes (N = 20 for each trial) and the proportion of germinated seeds was calculated each 24 h for a total of 96 h.

To determine the impact of the pollination treatment on the macro-nutritional composition of the pulses, a total of 50 seeds per treatment were finely ground using a laboratory mill (IK, Germany), and the powder obtained was freeze-dried to eliminate water residual content. The total starch and protein contents were analyzed. Specifically, the starch content was determined using the Total Starch Kit (Megazyme, Ireland) as described in Guzzetti et al., 2020, while proteins were extracted and quantified following the protocol described in Vaudo et al, 2018.

Untargeted metabolomics

The analysis of the metabolomic features of strawberries and cowpea seeds originating from the different pollination systems was carried out by exploiting analytical chemistry approaches.

Extraction of secondary compounds from strawberries

Prior to the extraction of the phytochemicals from strawberries, the fruits were completely freeze-dried to remove the aqueous component and then grinded to a fine powder. The extraction process was carried out by using a hydro-alcoholic solvent composed of MeOH 70% *v/v* and acidified at pH = 3.5 by using HCl 1 M. The drug-to-solvent ratio was equal to 1:20 *w/v* and each sample was macerated on an orbital shaker (Asal 711, Italy) for two extraction cycles lasting 15 minutes each. At the end of each cycle, the sample was centrifuged at 5000 g and the supernatant

was collected. The total extracts were evaporated up to dryness using a rotary evaporator (Steroglass, Italy) and then resuspended in 10 mL of ultrapure milli-Q H_2O.

Solid Phase purification of wild strawberry phytocomplexes

Due to the presence of a high concentration of sugars in the matrix, a solid-phase mediated purification was carried out to isolate the phytochemical fraction of strawberries. The process was carried out by exploiting Reverse Phase cartridges (Strata-X, Phenomenex, USA) which were loaded with the extracts obtained at Par. 2.4.1 and then eluted with a gradient spanning from 10% to 100% MeOH + 0.1% HCOOH v/v. The eluted fractions were kept at -80°C for the subsequent mass spectrometry analyses.

Phytochemical content

The phytochemical composition of the purified fractions of strawberries was tested for the total phenol content (TPC), Trolox Equivalent Antioxidant Capacity (TEAC), and total flavonoid content according to the protocols reported in Pioltelli & Guzzetti, 2023c.

Extraction of phytochemicals from cowpea seeds

Cowpea seeds were grinded to powder. The powder was extracted in a hydro-alcoholic solvent made of EtOH:H_2O 1/1 *v/v* for three extraction cycles lasting 10 minutes each in a drug/solvent ratio equal to 1:10 w/v with the support of a bath

sonicator (ArgoLab, Italy) with at room temperature and set at a frequency of 37 Hz. The extracts were evaporated to remove the organic solvents and stored at -80°C prior to the mass spectrometry analyses.

High-Resolution Mass Spectrometry (HRMS) analyses

The analysis of the metabolic features of both strawberry phytochemicals fraction and cowpea extracts was performed by using a Xevo-G2-qToF mass spectrometer (Waters, USA) according to the protocol described in Pioltelli & Guzzetti, 2023d. Briefly, the analysis was carried out by reverse-phase chromatography. Mobile phases were (A) H_2O + 0.1% HCOOH v/v and (B) MeCN + 0.1 % HCOOH v/v. The injection volume for each analysis ranged from 2 to 5 μL depending on sample concentration. The full scan analyses were performed both in positive and negative ion current and followed by MS^2 experiments for the identification of the phytochemicals responsive to the pollination treatments.

Statistical analysis

All the data were analyzed by R ver 4.3.1. Data originating from the morphometric analysis of both species were analyzed using an ordination approach based on Principal Component Analysis (PCA) followed by PERMANOVA analysis coupled with a post hoc Tukey analysis to evaluate the effect of the pollination treatment.

Packages exploited were vegan, ggplot2, and RVAidememoire. Data concerning the strawberries TSS, TA, pH, TSS:TA ratio, and number of fertilized achenes as well as those related to the proportion of aborted seeds and primary metabolism were analyzed by regression models with the pollination treatment as a fixed effect. Data about cowpea germination rates included as fixed effect the duration time of the experiment in interaction with the pollination treatment. Packages exploited were MASS, glmmTMB, and ggplot2. Details about the statistical distribution adopted are reported in Table S1.

Finally, the data obtained from the HRMS untargeted analysis were primarily processed on MS-Dial (version 4.9) for peak peaking, deconvolution, noise level setting, alignment, and normalization. These last steps were performed by exploiting the QC (Quality Control) runs performed in the full-scan experiments. Then, the normalized data were analyzed by PCA followed by PERMANOVA analysis coupled with a post hoc Tukey analysis to evaluate the effect of the pollination treatment. In case of significant differences among the pollination treatments, the most responsive metabolic features (log of their normalized areas) were investigated by an ANOVA test followed by a Tukey post hoc test. The significant ones were studied for chemical characterization. Their identification was performed by MS^2 experiments coupled with the consultation of both libraries of

natural products (EXPV17 on MS-Dial and The Waters Traditional Medicine on UNIFI) and literature research.

Results

Morphological traits

The morphological traits of both *F. vesca* and *V. unguiculata* were significantly affected by the pollination treatment. Specifically, in both cases, OP derived strawberries and cowpea seeds showed better parameters in terms of dimensions, colors, and viability compared to the SP and HP treatments and did not show any significant variation (Fig. 1A, B).

Analysis of the seed set

In *F. vesca* a higher number of fertilized achenes were observed in the OP treatment (Fig. 2A), and similar results were obtained for *V. unguiculata* which showed a higher number of seed per pod in the OP treatment (Fig. 2B). Furthermore, in cowpea pods, the proportion of aborted seeds was significantly lower in the OP treatment compared to SP ($p = 0.003$) while the HP treatment was characterized by an intermediate level both of fertilized achenes and aborted seeds. In addition, the germination rate of cowpea seed was significantly higher in the OP treatment, compared to SP and HP (Fig. S1)

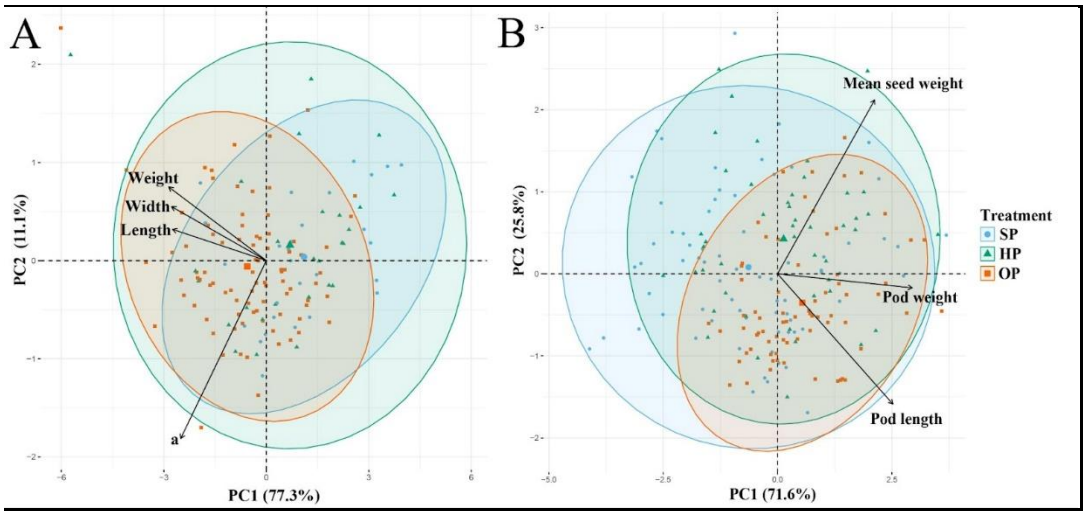

Fig. 1. output of the PCA performed on the morphological traits of *F. vesca* (A) and *V. unguiculata* seeds (B). The arrows indicate the direction where the specific variables tend to. In both cases, the OP treatment was characterized by bigger fruits and seeds (p = 0.002) compared to SP and HP which were found to be morphologically similar.

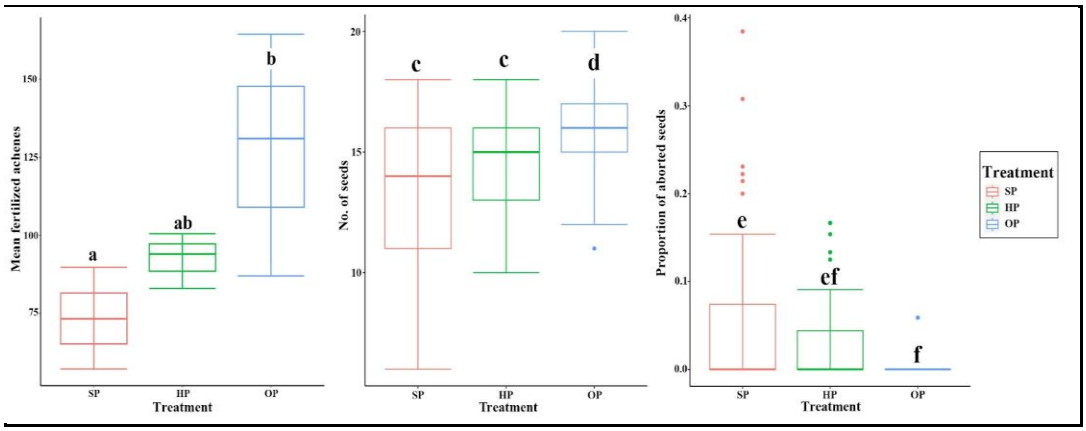

Fig. 2. Mean number of fertilized achenes per strawberry (A), number of seeds per cowpea pod (B) and proportion of aborted seed per pod (C). Different letters indicate the significance at the statistical level ($p < 0.05$).

Analysis of the commercial quality

In *F. vesca*, significant differences in TSS and the TSS:TA ratio were observed between SP and OP derived fruits. Concerning HP fruits. TSS was similar to OP while the TSS:TA ratio was similar to SP (Table 1). No significant differences among treatments were detected concerning TA and pH in strawberries and total starch and protein content in cowpea seeds (Table 1).

Table 1. Mean and SEM values of the quality parameters detected for the two investigated species. Different uppercase letters indicate variations relevant at the statistical level ($p < 0.05$).

Species	Quality parameter	SP	HP	OP
Fragaria vesca	TSS (%)	12.95 ± 0.24 [a]	11.07 ± 0.79 [b]	11.2 ± 0.94 [b]
Fragaria vesca	TA (%)	0.97 ± 0.25 [a]	0.86 ± 0.05 [a]	1.3 ± 0.35 [a]
Fragaria vesca	pH	3.55 ± 0.13 [a]	3.45 ± 0.06 [a]	3.47 ± 0.19 [a]
Fragaria vesca	TSS:TA	13.81 ± 2.55 [a]	12.72 ± 1.14 [a]	9.06 ± 1.79 [b]
Vigna unguiculata	TSC (%)	44.5 ± 3.24 [a]	45.28 ± 1.53 [a]	45.1 ± 0.6 [a]
Vigna unguiculata	TPC (%)	24.4 ± 0.18 [a]	24.49 ± 0.14 [a]	24.49 ± 0.12 [a]

Untargeted metabolomic investigation and identification of discriminant phytochemicals

Both the phytochemical fractions of strawberries and cowpea seeds were significantly affected by the pollination treatment. Results of the ordination analyses performed on the full scan traces are reported in Fig. 3. In detail, concerning the phytochemical investigations of strawberries, in positive ion current (Fig. 3B) a significant difference in the metabolic composition between SP and OP emerged (p = 0.018), while in negative ion current (Fig. 3A) OP derived fruits were different both from SP (p = 0.015) and HP (p = 0.033), which conversely did not show differences significant at the statistical level (p > 0.05). These differences were associated with a lower TPC and TFC in OP compared to SP and HP, while no significant differences among treatments emerged with respect to the TEAC (Table S3). The investigation of the phytochemical composition of cowpea extracts showed that SP seeds were significantly different both from HP (p = 0.006) and OP (p = 0.003) in negative ion current (Fig. 3C). Conversely, no significant differences (p > 0.05) among treatments emerged in positive ion current (Fig.3D). Table 2 shows the analysis of the discriminant phytochemicals contributing to the separation of the experimental groups in the multivariate analysis. In F. vesca many different secondary compounds were significantly higher expressed in OP treatment, among which anthocyanins, flavonoids, and ellagic acid derivatives were the most

common. In SP treatment, the untargeted metabolomic analysis revealed a significantly higher content of ellagitannins.

Concerning the differences observed in cowpea seeds, we found that most of the discriminant phytochemicals (mainly flavonoids, flavanols, and saponins) were significantly higher expressed in SP treatment, with the HP displaying an intermediate level of these metabolites, and the OP lower. Only two exceptions were observed since m/z 465.12 (characterized as taxifolin-O-hexoside) and m/z 505.21 (identified as an acetyl-hexoside of quercetin) were significantly higher in OP seeds compared to SP and HP.

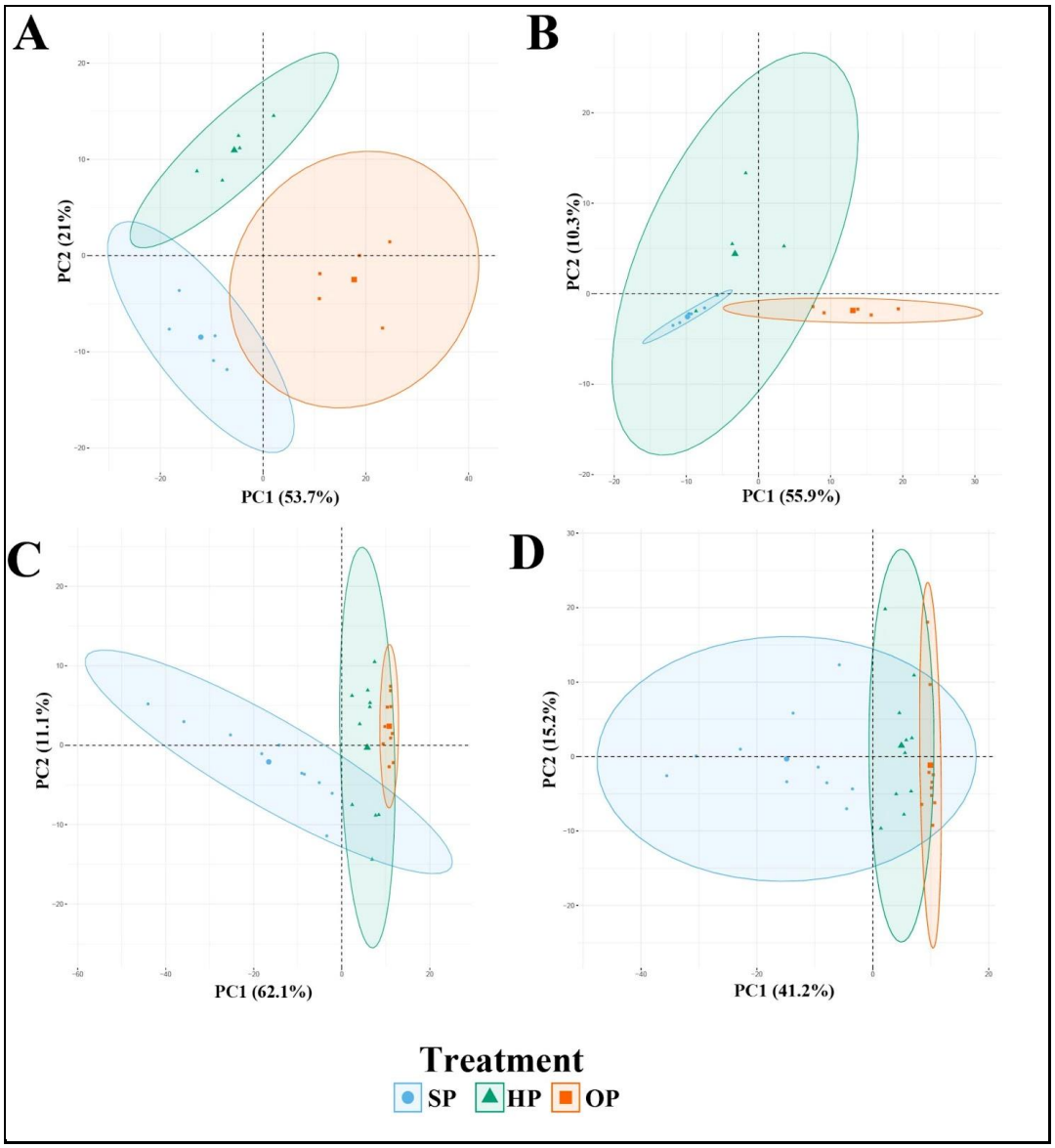

Fig. 3. Ordination analysis performed on the metabolic profiles of *F. vesca* phytochemical fractions in negative (A) and positive ion current (B) and on *V. unguiculata* extracts in negative (C) and positive ion current (D)

Table 2. Identification of the metabolites significantly involved in the differences among treatments. Rt = Retention time, m/z = mass to charge ratio. The symbols ">" and "<" indicate differences significant at the statistical level (p = 0.05), while "=" indicates non-significant differences.

Species	Rt	m/z	adduct	fragments	Ontology	ID	family	ref	stat
F. vesca	8.97	465.1	$[M-H]^-$	447, 285, 247	$C_{21}H_{22}O_{12}$	(2R,3R)-Taxifolin-3'-O-β-D-glucopyranoside	flavonoid	unifi	(SP = HP) < OP
F. vesca	14.5	491.2	$[M-HCOO]^-$	313, 161	$C_{21}H_{34}O_{10}$	(Z)-(1S,5R)-β-Pinen-10-il-β-vicinoside	monoterpene	unifi	(SP = HP) < OP
F. vesca	9.4	431.09	$[M-2H]^-$	269, 268, 224, 147	$C_{21}H_{20}O_{10}$	Pelargonidin-3-O-glycoside	anthocyanin	Sun et al., 2014	(SP = HP) < OP
F. vesca	12.58-13.13	461.0723	$[M-H]^-$	315, 301, 275	$C_{21}H_{18}O_{12}$	3-O-Methylellagic acid-3'-O-α-L-rhamnopyranoside	ellagic acid derivative	unifi	(SP = HP) < OP
F. vesca	11.36	567.21	$[M-HCOO]^-$	521, 359	$C_{26}H_{34}O_{11}$	Methylated flavonoid hexoside	flavonoid	Elshamy et al., 2019	(SP = HP) < OP
F. vesca	11.86	463.09	$[M-H]^-$	315, 300, 151	$C_{21}H_{20}O_{12}$	Myrcitrin	flavonoid	unifi	(SP = HP) < OP
F. vesca	16.87	255.07	$[M-H]^-$	213, 185, 171, 151, 145, 107	$C_{15}H_{12}O_4$	Pinocembrin	flavonoid	unifi + Ms_Dial	SP < HP < OP
F. vesca	10.29	371.1	$[M-H]^-$	249, 121	$C_{16}H_{20}O_{10}$	3-benzoyloxy-2-hydroxypropyl glucopyranosiduronic acid	phenolic acid derivative	MS-Dial	(SP = HP) > OP
F. vesca	14.94	549.16	$[M-H]^-$	255	$C_{26}H_{30}O_{13}$	Liquiritin apioside	flavonoid	MS-Dial	(SP = HP) < OP

F. vesca	12.86	315.01	$[M-H]^-$	300	$C_{15}H_8O_8$	3-O Methyl ellagic acid	ellagic acid derivative	unifi	(SP = HP) < OP
F. vesca	11.14	447.05	$[M-H]^-$	301, 300, 257, 229	$C_{20}H_{16}O_{12}$	Ellagic acid rhamnoside	ellagic acid derivative	Del Bubba et al., 2012	(SP = HP) < OP
F. vesca	6.12	203.08	$[M-H]^-$	142, 130, 116	$C_{11}H_{12}N_2O_2$	Tryptophan	amino acid	unifi	(SP = HP) < OP
F. vesca	8.58	447.15	$[M-H]^-$	285, 285	$C_{21}H_{20}O_{11}$	Kampferol-O-hexoside	flavonoid	Del Bubba et al., 2012	(SP = HP) < OP
F. vesca	9.38	449.11	$[M-H]^-$	355, 329, 287, 269, 193, 165, 137	$C_{21}H_{22}O_{11}$	Ferulic acid hexose derivative	phenolic acid	Sun et al., 2014	(SP = HP) < OP
F. vesca	8.67	331.1	$[M-H]^-$	127	$C_{14}H_{20}O_9$	Tetra-O-acetyl-dexoyhexoside	Sugar derivative	unifi	(SP = HP) < OP
F. vesca	6.93	947.05	$[M-H]^{2-}$	901, 883, 301	$C_{41}H_{24}O_{27}$	unknown ellagitannin	ellagitannin	Sun et al., 2014	(SP = HP) > OP
F. vesca	12.28	519.07	$[M-H]^-$	315, 300	$C_{23}H_{20}O_{14}$	Methyl ellagic acid acetyl hexoside	ellagic acid derivative	Sun et al., 2014	(SP = HP) < OP
F. vesca	11.43	477.06	$[M-H]^-$	301, 300	$C_{21}H_{18}O_{13}$	quercetin glucuronide	flavonoid	D'Urso et al., 2016	(SP = HP) < OP
F. vesca	8.17	291.09	$[M-H]^+$	161, 147, 139, 123	$C_{15}H_{14}O_6$	(+)Catechin	flavanol	MS-Dial	HP > SP > OP
F. vesca	10.01	433.11	M^+	271	$C_{21}H_{21}O_{10}$	Pelargonidin-3-O-glycoside	anthocyanin	Sun et al., 2014	(SP = HP) < OP
F. vesca	9.79	463.12	M^+	301	$C_{22}H_{23}O_{11}$	Peonidin 3-O-glycoside	anthocyanin	Del Bubba et al., 2012	(SP = HP) < OP
F. vesca	6.05	205.1	$[M-H]^+$	188, 170, 118	$C_{11}H_{12}N_2O_2$	Tryptophan	aminoacid	unifi	(SP = HP) < OP

F. vesca	17.2	246.24	$[M-NH4]^+$	124, 57	$C_{14}H_{28}O_2$	Ethyl laurate	fatty acid	unifi	SP < HP < OP
F. vesca	15.8	257.07	$[M-H]^+$	239, 215, 153, 131, 103, 77	$C_{15}H_{12}O_4$	Pinocembrin	flavonoid	unifi	SP < HP < OP
F. vesca	7.96	579.15	$[M-H]^+$	409, 287, 127	$C_{30}H_{25}O_{12}$	B-type procyanidin	procyanidin	unifi	SP < OP < HP
F. vesca	11.82	303.05	$[M-H]^+$	285, 257	$C_{14}H_6O_8$	Ellagic acid	ellagic acid	unifi	(SP = HP) < OP
V. unguiculata	451.1	2.4-2.7	$[M-H]^-$	289, 137, 109	$C_{21}H_{24}O_{11}$	Catechin-O-glucoside	flavanol	Zhao et al., 2013	SP > HP > OP
V. unguiculata	289.1	3.9	$[M-H]^-$	123, 109	$C_{15}H_{14}O_6$	Catechin	flavanol	MS-Dial	SP > HP > OP
V. unguiculata	385.1	3.58	$[M-HCOO]^-$	134, 85	$C_{15}H_{16}O_9$	Caffeic acid derivative	phenolic acid derivative	unifi	SP > HP > OP
V. unguiculata	493.15	4.05	$[M-H]^-$	289, 245, 203	$C_{23}H_{26}O_{12}$	Catechin-O-glucoside-O-acetoside	flavanol	Zhao et al., 2013	SP > HP > OP
V. unguiculata	465.12	4.97	$[M-H]^-$	285, 151	$C_{21}H_{22}O_{12}$	Taxifolin-O-hexoside	flavonoid	unifi	(SP = HP) < OP
V. unguiculata	567.23	5.53	$[M-HCOO]^-$	521, 506, 359, 344, 217	$C_{26}H_{34}O_{11}$	Neolignan	Lignan	unifi	SP > HP > OP
V. unguiculata	505.11	6 .28	$[M-H]^-$	463, 301, 300	$C_{23}H_{22}O_{13}$	Quercetin-O-(acetyl-hexoside)	flavonoid	unifi	(SP = HP) < OP

V. unguiculata	625.16	5.16, 5.3	$[M-H]^-$	301, 300	$C_{27}H_{30}O_{17}$	Quercetin-di-O-hexoside isomers	flavonoid	MS-Dial	SP > HP > OP
V. unguiculata	957.54	10.55	$[M-H]^-$	615, 263, 221	$C_{48}H_{78}O_{19}$	3-Glucose-Galactoside-Glucurunate Soyasapogenol B	Triterpene saponins	MS-Dial	SP > HP > OP
V. unguiculata	663.33	7.64	$[M-H]^{2-}$	-	-	uknown peptide	peptide	see text	SP > HP > OP
V. unguiculata	677.32	7.81	$[M-H]^{2-}$	-	-	uknown peptide	peptide	see text	SP > HP > OP
V. unguiculata	648.32	7.93	$[M-H]^{2-}$	-	-	uknown peptide	peptide	see text	(SP = OP) > HP
V. unguiculata	640.32	8.01	$[M-H]^{2-}$	-	-	uknown peptide	peptide	see text	SP > HP > OP
V. unguiculata	327.23	9.17	$[M-H]^-$	211, 183, 125	$C_{18}H_{32}O_5$	Trihydroxy-octadecadienoic acid	polyunsaturated fatty acid	unifi	SP > HP > OP
V. unguiculata	987.54	9.13, 9.86	$[M-HCOO]^-$	941, 733, 615, 457	$C_{48}H_{78}O_{18}$	Azukisaponin isomers	Triterpene saponins	unifi	SP > HP > OP
V. unguiculata	955.52	11.23	$[M-H]^-$	613, 455	$C_{48}H_{76}O_{19}$	Soyasaponin	Triterpene saponins	unifi	SP > HP > OP
V. unguiculata	985.54	11.45	$[M-HCOO]^-$	921, 613, 455	$C_{48}H_{76}O_{18}$	Dehydrosoyasaponin	Triterpene saponins	unifi	SP > HP > OP

Discussion

This study represents a pioneering investigation demonstrating the influence of pollination mechanisms on the nutritional and commercial quality of representative crops relevant to human health. While existing research has extensively elucidated pollination's role on crop yield and commercial quality, its influence on the qualitative and quantitative aspects of nutritional quality has been largely neglected in terms of dedicated experimental studies so far. Specifically, our findings provide the first empirical evidence of coherent variations in the morphology, reproductive success, and phytometabolite composition of two very distinct crops, significantly enhancing the comprehension of the relationship between pollination and crop metabolism, with putative consequences on the human value of derived foods.

In recent years, a handful of studies have investigated macro- and micronutritional variations in crops in response to pollination (Brittain et al., 2014; Klatt et al., 2014, Wietzke et al., 2018), yet none have looked at the secondary metabolites, except for a recent study by Schurr et al. (2022), focusing on anethol concentration in fennel that found a significantly higher biosynthesis of this compound in fruits exposed to insect pollination compared to self and hand pollinated ones. Although the observed metabolomic variations that we documented cannot be directly

translated into nutritional improvement, they hold significant importance as they contribute valuable information to the scientific landscape. Their importance particularly emerges in a context where the impact of pollination is not yet definitely defined, to the extent that a recent meta-analysis attempted to clarify this topic and indicated that while pollination affects quality and organoleptic properties, its influence on nutritional value is far more evident than expected.

The relevance of the present study is also supported by the fact that two distinct plant species with different fruit types, *F. vesca* having fleshy fruit, and *V. unguiculata* dry ones, were chosen as experimental models to represent crops that are both phylogenetically distant and characterized by different levels of reliance on animal pollination for reproduction. While *V. unguiculata* is a pulse that shows a high rate of self-pollination (> 95%), *F. vesca* produces fleshy fruit more dependent on cross-pollination to be productive (> 20%). Interestingly, both species exhibited similar responses to pollination treatments both at the morphological and metabolic levels, suggesting that insect pollination impact is not restricted to species or cultivars relying heavily on cross-pollination.

The observed improvements in seed set and marketable quality in both *F. vesca* and *V. unguiculata* are consistent with previous research. Yet, the underlying mechanisms guiding these variations remain poorly disentangled. Likely, they are determined by phytohormonal processes activated by the fertilization success

(Wietzcke et al., 2018). This hypothesis, already partially investigated in other studies (e.g., Rosiansky et al., 2016) is supported by our results. The fertilization success estimated in the two species was higher in the OP treatment with a significantly higher number of fertilized achenes in *F. vesca* and increased seed set and seed viability in *V. unguiculata*. The higher efficiency of animal pollination compared to self and hand pollination has already been observed in other similar studies (Garratt et al., 2014, Wietzke et al., 2018) and is a crucial parameter to consider when interpreting the results obtained. Indeed, fertilization success is directly linked to phyto-hormonal pathways as indicated by the study of Wietzcke et al., 2018 in which the concentration of auxin in the first phases of fruit development was significantly correlated with the proportion of fertilized achenes. Endogenous auxins play primary roles in the receptacle and seed development, also mediating the synthesis of other phytohormones such as gibberellins, guiding seed formation and fruit growth (Dorcey et al., 2009).

According to the recent conclusion of Gazzea et al., 2023 we found that the primary metabolism (i.e., starch and protein content) in *V. unguiculata* seeds was similar in all the investigated treatments. This lack of an effect can be since these nutritional classes are more affected by other factors, such as genetic, environmental conditions, or agronomic practices (Dumas et al., 2003; Marini et al., 2015). Another hypothesis is that the standardization of the macronutrients concentration among

the different treatments is guided by a dilution effect (Gazzea et al., 2023) that follows the rapid cell expansion and the increase of water content in OP fruits and seeds (Garratt et al., 2014; Samnegård et al., 2019), that are indeed characterized by an increase in size and weight.

Our findings have significant implications both for agriculture and the food industry, as well as ecological implications. In the context of increasing global regulations on food quality, suboptimal food production resulting from inadequate pollination service poses a challenge for growers. This study underscores the essential role of animal pollination in enhancing crop quality, and ultimately their economic value, potentially encouraging the adoption and implementation of mitigation strategies, such as the creation of flower strips and provision of artificial nesting resources for insect pollinators in agricultural environments. Despite the adoption of these good practices represents a cost for the grower, considering the results obtained here and in the other studies, these interventions could ultimately translate into added economic value. From the ecological perspective, the observed variation in the metabolome of fruit and seed sheds new light on the field of ecological interactions. The presence of particular compounds is a determinant in mediating ecological interactions. The color of the receptacle is indeed considered an honest signal that allows birds to assess the nutrient status of berries (Lomáscolo et al., 2010; Schaefer et al., 2014). By considering this, the results we obtained for *F. vesca*, where OP

fruits were characterized by a higher redness, related to the higher concentration of compounds belonging to the anthocyanins group, have important implications for frugivory and ultimately seed dispersal. Not only anthocyanins but also many other phytochemicals belonging to the classes of flavonoids and ellagic acid derivatives have been found to be elicited in strawberries originating from the OP treatment.

Some of the phytochemicals detected in the OP treatment are known to act as strong antioxidant agents, thus representing a valuable source of nutraceutical compounds for the human diet. Conversely, a class of polymers, the ellagitannins, occurs more in SP and HP derived strawberries compared to OP fruit. This may be at the basis of the higher TPC and TFC of the analyzed extracts since the reaction guiding these colorimetric assays mainly depends on the number of hydroxyl groups of a compound, and tannins are polymers endowed with a very high number of hydroxyl units. However, the palatability of the fruit may be endangered by a too high level of these phytochemicals, since they are highly involved in conferring astringency to foods, therefore worsening their overall value. In cowpea seeds, the OP treatment was found to exhibit lower concentrations of phytochemicals compared to HP and, particularly, SP. The only exception regarded two flavonoid derivatives that showed higher relative concentrations in OP compared to the other treatments. Despite being a source of interesting phytochemicals, from a dietary

perspective it is important to underline that pulses such as cowpea seeds need to be cooked (mainly boiled) to be consumed. Since most of the phytochemical components are extracted in the cooking water, it should be interesting to evaluate whether these differences are still maintained after the cooking process, with putative consequences at the dietary level. Moreover, some of these compounds, such as saponins, act as anti-nutrients, therefore, it is arguable that a lower content in food (as in the case of OP seeds) may be associated with better digestibility. From an ecological point of view, the occurrence of secondary compounds in plant tissues is usually associated with stress conditions, as it is intendable by interpreting the results showing the lower germination ability of SP and HP treatment compared to OP ones. It is arguable that the pollination events occurring in SP and HP may be affected by an incomplete deposition of pollen on the flower stigmas, which ultimately impacts the germination ability and the phytochemical profile of the derived seeds, generating stress phenomena to the embryo development. Therefore, considering all these aspects, we can conclude that seeds and fruits produced by cross-fertilization induced by insect visitation could possess a fitness advantage compared to the self-pollinated ones. If it can be supposed that shared phytohormone pathways drive responses in fruit commercial quality and nutritional metabolic profile, investigating the underlying biological mechanisms represents a promising research frontier. Future studies should deepen the

investigation of these processes by looking for example at differential gene expression in the different phases of fruit and seed development after pollination treatment through a transcriptomic approach. Furthermore, the investigation of possible communication pathways between the insect and the plant can be very interesting, as recent studies have indeed demonstrated how plants can sense airborne sound produced for example by flying insects and can respond to this stimulus by increasing sugar concentration in the nectar (Veits et al., 2019). Therefore, how this communication translates at the fruiting stage remains to be understood.

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SUPPORTING INFORMATION

Table S1. details about the variables analyzed through regression approaches based on Generalized Linear Models (GLMs)

Species	Variable analyzed	Unit of measurement	Statistical distribution adopted in GLM
Fragaria vesca	Number of fertilized achenes	-	Poisson distribution
Fragaria vesca	TSS, TA	%	Binomial distribution
Fragaria vesca	pH	-	Gaussian distribution
Fragaria vesca	TSS:TAA	-	Gaussian distribution
Fragaria vesca	TPC, TFC, TEAC	mg / mg	Binomial (or alternatively quasibinomial/ beta) distribution, depending on the overdispersion parameter
Vigna unguiculata	Number of seeds per pod	-	Poisson distribution
Vigna unguiculata	Proportion of aborted seeds per pod	-	Binomial distribution
Vigna unguiculata	TSC, TPC	%	Binomial distribution

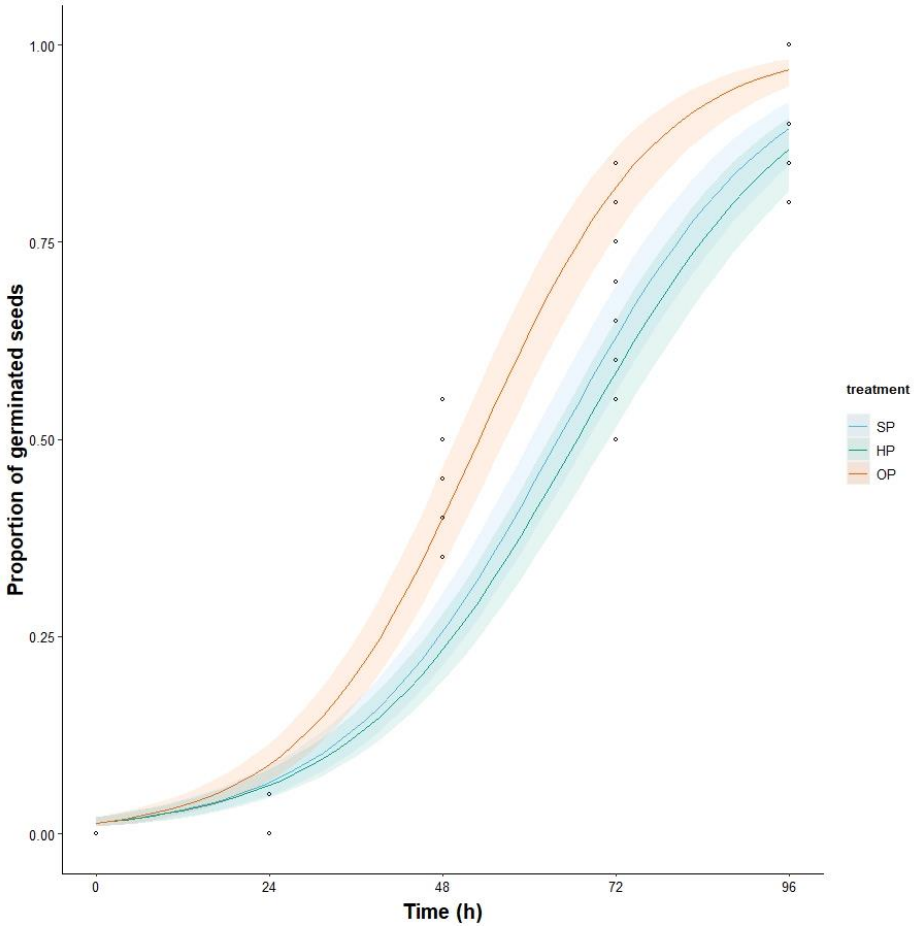

Figure S1. Proportion of germinated seeds along a 4-day experiment comparing the different pollination treatments (*N*=60). SP = self-pollination, HP = hand pollination, OP = open pollination. Significant differences among treatments occur when the 95% confidence bands do not overlap.

Table S3. Total phytochemical content (expressed as % w/w) of strawberries originating from the different pollination treatments. Values are reported as mean ± SEM. Significant differences ($p < 0.05$) among treatments are indicated by different uppercase letters. TPC = Total Phenol Content, TFC = Total Flavonoid Content, TEAC = Trolox Equivalent Antioxidant Capacity.

	TPC (%)	**TFC (%)**	**TEAC (%)**
SP	2.324 ± 0.092 [a]	0.823 ± 0.031 [a]	4.835 ± 0.444 [a]
HP	2.444 ± 0.053 [a]	0.843 ± 0.016 [a]	4.772 ± 0.225 [a]
OP	1.947 ± 0.054 [b]	0.678 ± 0.015 [b]	3.817 ± 0.372 [a]

Part III

Conclusions and Final remarks

Conclusions

Through the application of innovative methodologies, spanning multiple disciplines and under both controlled and natural experimental conditions, this project has illuminated previously unexplored dimensions of pollination ecology. The most relevant result concerns the definition of how anthropogenic disturbances impact the quality of flowering resources and the nutritional ecology of pollinator insects, as well as the influence of pollination processes in shaping the characteristics of seed and fruit food items. Therefore, the findings obtained in this project shed new light on the intricate relationships between land use intensification and functional biodiversity, offering new strategies for achieving restoration ecology purposes, up to supporting human food security.

To enhance the significance of the results and implications, from both a scientific and managerial perspective, some 'take home messages' have been drafted. If properly adopted these messages could have a substantial impact on the monitoring, conservation, and restoration of urban and peri-urban biodiversity, especially in areas where human disturbance is extensive, multifaceted, and continuously increasing.

Message 1. Efforts in biodiversity monitoring should be specifically directed towards analyzing quantitative and functional aspects, rather than solely focusing

on species composition. Many existing biodiversity estimation tools are mainly adopted to assess species diversity, the occurrence of rare, endemic taxa, and of focal/flagship species. Although these assessments are relevant to implement conservation strategies, they do not provide a comprehensive and realistic understanding of an ecosystem biodiversity.

Methodological and theoretical advances have emerged from the different case studies (Case study I and II) addressed during this doctoral research project. These findings underscore the shared necessity of defining standardized monitoring methodologies. This urgency cannot be overstated, especially considering recent international and large-scale initiatives focused on biodiversity assessment, such as the EGD and the Nature Restoration Law. Methodological coherence will establish a foundation of reliable and shareable knowledge, upon which policymakers and stakeholders will base and adjust their interventions. In addition to providing advances in scientific knowledge, the main requisite is that research in this field should be characterized by standardization in applying multidisciplinary and transdisciplinary approaches, with the final aim of comprehensively investigating environmental, animal, and human health issues.

Message 2. The analysis of biodiversity demands a multidimensional and multiparametric approach. It is increasingly essential to evaluate, under an

integrative framework, the impacts of human pressures on biological resources and their consumers. This concept emerges clearly from Case study III, which delves into investigating the importance of environmental quality in fostering functional biodiversity (i.e., nutritional quality of nectar and pollen foraged by insects).

The results obtained indicated that landscape anthropization influences chemical composition of floral rewards. These findings challenge the notion that the nutritional landscape for pollinator communities can be solely defined by the quantity and availability of flower resources and demonstrated that the impact of urbanization on pollinators diet is not only determined by constraints to pollinator foraging choice but is also shaped by the effects that land use displays on plant metabolism. Furthermore, the importance of the community-level investigation here adopted is further confirmed by the observed species-specific response to anthropogenic pressures. This phenomenon highlights the need for studies that encompass the entire community, and not a single model species.

Additional investigation on flower resources chemical variability can provide early warning systems of plant stress exposure and inform about which species are more resilient to anthropization-related pressures. This knowledge can provide valuable practical insights for the implementation of targeted mitigation strategies. The creation of a reference database of pollen and nectar nutritional content encompassing many species, coupled with information on specific responses to

environmental conditions, can actually constitute a valuable benchmark to determine which species have the most potential for supporting pollinator communities in a particular area or ecoregion. This information will be crucial for planning tailored Nature Based Solutions (NbS), such as the creation of green areas in cities or the creation of flower strips in the agricultural ones. The availability of floral rewards nutritional data at the community level can also complement large-scale monitoring projects of meadows resources. Indeed, in recent years, given the continuous innovation and the reduced costs of monitoring technologies, many programs have been launched that use remote-sensing derived data from satellite images or obtained by using UAV to describe the composition and abundance of plant communities, even within areas of reduced surface. If coupled with data about the species-specific nutritional profile, this strategy can revolutionize our approach to the characterization of floral landscape and its potential in supporting the pollination ecosystem service.

From an ecological perspective, the observed variations in the nutritional profile of pollen and nectar in urban and agricultural habitats may impact plant-pollinator interactions and act synergistically with the other pressures these habitats exert on pollinator communities. Such variations can ultimately determine shifts in the quality of the pollinators' diet, exacerbating the already deep impact of habitat fragmentation observed in Chapter II.

Message 3. Aiming at achieving successful ecological restoration through the accurate planning of suitable mitigation strategies, it should be considered that the creation of green habitats is not sufficient to guarantee functional and resilient ecosystems. For example, implementing the connectivity between green areas in urban and agricultural landscapes is a crucial aspect to create ecological corridors, provide trophic niches and prevent biodiversity loss. Specifically, for the creation of suitable habitat for pollinators, some functional aspects (e.g., foraging behaviour) should be considered, as highlighted by Case study IV.

In the second chapter, the focus shifted from plants to insects, and specifically, on a polylectic, eusocial pollinator species, *B. terrestris.* The urban landscape of Milan (NW Italy) served as an open-air laboratory to investigate the impact of habitat fragmentation and local floral resources availability on the nutritional ecology of this species. Increased fragmentation significantly affected the diet quality by reducing the protein content and altering the protein/lipid ratio. The nutritional profile of the pollen also showed tight association with its taxonomic composition. Overall, these results clearly indicate a constrained foraging behavior as the urban matrix becomes more fragmented, confirmed also by the recording of longer foraging trips at sites characterized by lower degree of green cover. The impoverishment of the diet associated with increased green cover fragmentation and decreased richness of flowering plants raised serious warnings on the potential

of urban green areas to sustain local pollinator populations. The reduction in the concentration of protein in the pollen pellet and the unbalancing of the ratios between macronutrient composition can indeed determine significant reduction in the overall fitness of the colony by impacting the survival rate, development, and reproductive success. As well as in Chapter I (Case study III), these results underline how the urban landscape could have an impact on the whole community of pollinators and thus on the potential effects on the ecosystem services they provide. Filling this gap constitutes a step forward for the designing and management of urban green spaces, increasing their contribution to regional and global biodiversity conservation.

Message 4. Currently, the recognition of the contribution of animal-mediated pollination to food security relies only on the enhancements in terms of yield and commercial quality. Chapter III opens new frontiers in the investigation of pollination effect on crop quality, highlighting the need to also look at the micronutritional components that have important implications for food security and human health, thus underlying the role of pollinator insects in shaping not only quantitative, but also the qualitative traits of food items on our table.

Case study V significantly advanced our understanding of insect-based pollination in food production and security. While previous studies primarily focused on the impact of entomogamous pollination on crop yield and commercial quality, the

chemical features of fruit and seeds, especially concerning secondary metabolites, has been largely overlooked. On the other hand, these compounds occur in plant-based products in smaller amounts compared to macronutrients, play key roles in mediating ecological interactions and generally, they have positive implications for human health. For instance, they influence fruit color, affecting frugivory and seed dispersal, and contribute to the aroma and flavor of foods of food items, thereby influencing their commercial quality. Many phytochemicals also show bioactivity and can contribute for example to mitigate several metabolic syndromes. The findings obtained in Case study V through an untargeted metabolomic approach gave first evidence of the role of pollination mediated in shaping the phytochemical composition of fruit/seeds, with a plethora of secondary metabolites endowed with nutraceutical properties (e.g., anthocyanins, flavonoids, ellagic acid derivatives) occurring in significantly higher proportion when deriving from insect-mediated pollination events in wild strawberries, while in cowpea seeds the majority of the phytochemicals detected (among which many anti-nutrients, such as triterpenic saponins) was found at higher concentrations in self-pollinated seeds, probably due to stress phenomena occurring at the embryos development.

However, many open questions persist on the mechanisms underlying these changes. First, the metabolic pathways underlying these processes have not been characterized yet. Preliminary efforts have recently been made in this direction,

such as the research conducted by Lama et al. in 2020, who explored gene expression patterns through a transcriptomic approach in *Ficus carica*. They observed that many genes involved in the organic acid metabolism were significantly over-expressed in pollinated fruit compared to parthenocarpic ones. Secondly, the possibility of a direct communication between insects and the pollinated plant have been hypothesized, marking a new frontier in pollination ecology. While plant's ability to sense their environment to light stimuli (Chory, 2010), touch (Monshausen & Hasweel, 2013) and vibrating (De Luca & Vallejo-Marín 2013) are well documented, the capacity of plants to respond to airborne sound, as the one emitted by insect in flight, has only recently been demonstrated (Veits et al. 2019). The potential influence of such a biophysical interaction on crop quality is still to be explored. The groundbreaking potential of conducting experiments like those presented in Chapter III, including exposure to playback sounds of flying insects, can revolutionize this field, particularly for agricultural applications. Ultimately, the investigation of the link between pollination services and human nutrition and health is still at its dawn and most of the current knowledge is based on studies that investigated a handful of crops and mostly focused on macronutrients (e.g., proteins, lipids, and carbohydrates) relevant to crop marketability.

Final remarks

Overall, the case studies addressed in this PhD project highlighted the critical role of habitat integrity and health in mediating the human health benefits derived from pollinators. The key findings emphasize the importance of maintaining and creating green areas within urban and agricultural environments. The adoption of Nature-based Solutions that will mitigate habitat loss and fragmentation phenomena by increasing the amount of suitable habitat for pollinators and strengthening the connectivity among these is crucial. New land use policies fostering the creation of pollinator-friendly habitats represents a vital step towards sustainable agriculture and urban development. Furthermore, the results presented can ultimately help to lighten the interest of stakeholders, academia, and civil society in promoting biodiversity conservation, ensuring sustainable food production, and facilitating ecological restorations as pivotal steps toward a more sustainable development of human-transformed landscapes. Multidisciplinary collaborations among ecologists, health and social scientists, policymakers and local stakeholders is essential to further develop comprehensive research approaches and to develop effective conservation policies aimed at promoting the synergy between human and environmental health. Especially in urban environments, cities offer unique opportunities for public involvement, fostering projects based on citizen science that can significantly raise public awareness on this critical topic.

In conclusion, embracing a holistic perspective on the interconnections between human societies, natural capital, and ecosystem services is crucial. This approach aligns with the foundation principle of ecological economics, underscoring the importance of the symbiotic relationship between human and nature.

Appendix

During this PhD, I contributed to other studies related to the field of pollination biology. I also contributed to other investigations, not directly related to this issue. A list of these articles, starting with those more closely related to the issues treated in this thesis, is reported below.

1. Tommasi Nicola, **Pioltelli Emiliano**, Biella Paolo, Labra Massimo, Casiraghi Maurizio, Galimberti Andrea (2022). Effect of urbanization and its environmental stressors on the intraspecific variation of flight functional traits in two bumblebee species. *Oecologia*, *199*(2), 289-299.

Contribution: In this study, I contributed to the conceiving of the idea and the designing of the methodology. I actively participated in field activities, conducted statistical analyses, and contributed to the writing of the manuscript.

2. Biella Paolo, Tommasi Nicola, Guzzetti Lorenzo, **Pioltelli Emiliano**, Labra Massimo, Galimberti Andrea (2022). City climate and landscape structure shape pollinators, nectar, and transported pollen along a gradient of urbanization. *Journal of Applied Ecology*, *59*(6), 1586-1595.

Contribution: In this study, I contributed to the collection of data and to the revision of the manuscript.

3. Tommasi Nicola, Colombo Beatrice, **Pioltelli Emiliano**, Biella Paolo, Casiraghi Maurizio, Galimberti Andrea (2023). Urban habitat fragmentation and floral resources shape the occurrence of gut parasites in two bumblebee species. *Ecology and Evolution*, 13, e10299.

Contribution: In this study, I contributed to field activities and in the review and editing of the manuscript.

4. Scaccabarozzi Daniela, Guzzetti Lorenzo, **Pioltelli Emiliano**, Brundrett Mark, Aromatisi Andrea, Polverino Giovanni, Vallejo-Marin Mario, Cozzolino Salvatore, Zong-Xin Ren. (2023). Introduced honeybees (*Apis mellifera*) in orchid pollination: surrogate pollinators or pollen wasters?

Contribution: In this study, I conducted the statistical analysis and contributed to the review and editing of the manuscript.

5. **Pioltelli Emiliano**, Sartirana Chiara, Copetta Andrea, Brioschi Maura, Labra Massimo & Guzzetti Lorenzo (2023). *Vigna unguiculata* L. Walp. Leaves as a Source of Phytochemicals of Dietary Interest: Optimization of Ultrasound-

Assisted Extraction and Assessment of Traditional Consumer Habits. *Chemistry & Biodiversity*, e202300797.

Contribution: In this study, I contributed to the conceptualization and design of the experimental setup. I contributed also to the data collection and curation, the statistical analysis, and the writing of the manuscript.

6. Nasuelli Martina, Ilahiane Luca, Boano Giovanni, Cucco Marco, Galimberti Andrea, Pavia Marco, **Pioltelli Emiliano**, Shafaeipour Arya, Voelker Gary, Pellegrino Irene (2021). Phylogeography of *Lanius senator* in its breeding range: conflicts between alpha taxonomy, subspecies distribution and genetics, *The European Zoological Journal*, 89:1, 941-956.

Contribution: In this study, I contributed to the extraction and sequencing of DNA, to the phylogenetic analysis, and to the reviewing and editing of the manuscript.

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Contribution: In this study, I contributed to the data collection and to the writing and editing of the manuscript.

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