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**DISENTANGLING THE EFFECTS OF HUMAN-ALTERED
ENVIRONMENTS ON POLLINATORS AND THEIR
INTERACTION WITH PLANTS: AN INTEGRATIVE ASSESSMENT**

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ABSTRACT

The process of pollination is a fundamental aspect that ensures both wild and cultivated plants reproduction and it is very relevant for the ecosystem services provided to humans. Pollination by animals is very common in nature (especially by Hymenoptera and Diptera) and it has been shown that it is of particular importance for maintaining ecosystem resilience and for human wellbeing. Despite their well-known importance, scientists have reported a worrying pollinator decline at a global scale. Multiple threats, mainly related to anthropogenic activities, have been identified as drivers of this biodiversity loss. Among these, land use intensification has been recognised as the most important one. The world's growing population has been driving a rapid conversion of natural and semi natural habitats into anthropogenic landscapes, such as urban and agricultural areas. Such processes are altering landscape composition, configuration, but also the associated environmental features among which the microclimate conditions rank first. As a consequence, direct and indirect factors are shaping the pollinator communities, ranging from biodiversity loss to altering of plant-pollinator interactions, with severe implications on the overall pollination service.

In the context of this PhD thesis the effect of land use intensification, specifically features related to landscape composition and configuration have been investigated at different levels (e.g., species, community, and interactions) in order to address both fundamental and applicative issues. To reach this goal, a multidisciplinary approach has been taken: Field data collection has been integrated with GIS-based approaches to describe land use and also local features; laboratory analyses, such as morphometric evaluations, analysis of pollen deposited on flower's stigmas and taxonomic identification through DNA-based tools (e.g., DNA metabarcoding) have been adopted to assess variations on pollinator biodiversity, their functional traits, the interactions with plants, and on the pollination efficiency. Plant-pollinator network indices have been widely used to compare mutualistic interactions from contrasting environmental conditions. Moreover, different statistical approaches have been applied adapting the selected strategies to the nature of the investigated variables.

In this PhD thesis, the fundamental scope was to provide advice for land use management policies easily transferable in multiple contexts. This major purpose

has been achieved through intermediate goals disentangled in different study cases that took place in very different ecological contexts, covering three different continents, Sub-Saharan Africa (Tanzania), Maldivian islands (Faafu and Dhaalu atolls), and North Italy (Milan metropolitan city).

The first study case investigated the effect of urbanization and agricultural land uses on pollinators and plants inhabiting smallholder farms of Tanzania. Results indicated that pollinator richness decreases with the proportion of urban and agricultural land. Moreover, the analysis of plant-pollinator interactions highlighted an increased competition for resources among individuals in response to the same drivers of land use change. Furthermore, at the local scale, the floral resources availability reversed the negative effects posed by anthropic land use, increasing species richness, and reducing competition. This encourages the adoption of nature-based solutions (e.g., planting flower strips and hedgerows) to create suitable environmental conditions for pollinators.

In the second study case the effects of changes in land use configuration, and specifically the increasing green habitat fragmentation, have been investigated in Maldivian islands, considered as simplified and isolated ecological model systems. Results indicated that low degrees of green areas fragmentation in anthropic habitats promote pollinator richness but reduce the complexity of the observed plant-pollinator interactions, a condition that may reflect lower pollinators functional redundancy. Moreover, the pollination efficiency seemed to be indirectly altered by fragmentation, since higher species richness resulted in improved pollination success in sentinel plants. These results prove that fragmentation could affect both pollinator insects biodiversity and their foraging strategy, with different responses depending on the intensity of this phenomenon. Conservation efforts to promote and maintain high pollinator biodiversity must be encouraged to ensure an efficient pollination service.

In the third study case pollinators communities along a gradient of urbanization in the metropolitan area of Milan have been investigated, confirming that low degree of disturbance (i.e., low proportion of surface covered by concrete and infrastructures or low degrees of green area fragmentation) may benefit pollinators by increasing their abundance. However, a general negative effect can be associated with growing degrees of disturbance. Pollinators have also been found to collect floral resources from less species in the more urbanized areas, confirming

the previously observed simplification of plant-pollinator interaction networks in the more anthropized landscapes. Moreover, flower resources also shape their nutritional content in response to land use composition, with increasing sugar content in the nectar observed in contexts characterized by higher impervious coverage in the surrounding (e.g., concrete and infrastructures).

The fourth case of study is focused on pollinator morphological response to land use alteration. Microclimate variation in urban landscapes, specifically the heat island resulting from increased proportion of impervious surfaces, have been proved to shape morphological functional traits (i.e., body size and wing asymmetry) in two bumblebee model species. Both species were negatively associated with high urban temperatures but showed idiosyncratic responses. Specifically, *Bombus pascuorum* reduced body size without increasing wing asymmetry in response to temperature increases. Conversely, *B. terrestris* body size remained constant, while wing asymmetry increased with temperatures. These results indicates that the microclimate condition associated to urban landscape deeply impact these species, since the here highlighted variation in functional traits can be directly related to a reduction in the dispersal ability of these species.

In the last study case, in order to refine the pollen DNA metabarcoding pipeline, I critically reviewed a step of the existing post-sequencing process. It has been proved that despite the increasing application of pollen DNA metabarcoding for the study of plant-pollinator interactions, a lack of shared methodology exists. Specifically, various approaches have been previously used to manage pollen DNA metabarcoding data. In this context, different filtering strategies could be used on high throughput sequencing reads to remove false positives and/or rare interactions. In this work I proved that adopting different strategies to manage DNA metabarcoding data leads to a significant variation in the ecological interpretation of results, and in particular to changes in plant-pollinator interaction indices. These results have been used to provide guidance in the application of this molecular tool in ecological studies.

Overall, the results obtained throughout this PhD thesis provided new evidence that could be followed for future design of urban landscapes but also to adopt local scale solutions to create pollinator-friendly conditions in anthropized landscapes. In particular, the results presented here suggest that the maintenance of green spaces should be fostered in land use planning, to reduce the impact posed by land

use intensification on pollinators while mitigating the creation of non-favourable microclimatic conditions. Moreover, the interconnection among green patches should be encouraged along with the adoption of nature-based solutions necessary to promote pollinator health and biodiversity. Policy makers should consider these and other scientific research outcomes in the future and integrate them into management actions. Indeed, as well proposed by the “One health “concept, human health can be ensured only by preserving the health of ecosystems in all their parts.

Keywords: Landscape ecology, Landscape anthropization, Pollination ecosystem service, Pollinator biodiversity, Plant-pollinator Interactions.

1. ECOSYSTEM SERVICES AND POLLINATION

Ecosystem services have been defined as all those benefits that humans derive from the ecosystems (Millennium ecosystem assessment (MA), 2005). Although the magnitude of all these benefits is difficult to estimate, their economic value is supposed to reach \$125 trillion (USD)/year (Costanza et al., 2014). These multiple gifts provided by nature have been classified in at least four main categories called provisioning, regulating, supporting and cultural services. Briefly, the first one refers to the provision of goods as food, water, and more in general raw material. The supporting services encompasses those processes as the nutrient cycles or primary production, while the cultural services are immaterial and refers to the contribution of the natural environment to the mental and spiritual wellbeing. The regulating services, at last, are deputed to the moderation of natural phenomena and include for instance the processes of climatic regulation, water purification, and organic matter decomposition (IPBES, 2016).

Among the regulating ecosystem services, pollination is one of the most important as it is deeply involved in food production and thus in the maintenance of human wellbeing. It consists in the process of pollen transport between flowers, that is necessary to promote sexual union of plants gamete, and therefore, reproduction. Although several plants rely on wind for the transport of pollen, this process is promoted by animals in up to 90% of flowering plants (Buchmann and Nabhan, 1996, Ollerton et al., 2011). Animals visit flowers looking for food, specifically nectar and pollen (Venjakob et al., 2021), and while feeding on flowers, they promote pollen dispersion. Given these assumptions, animal-mediated pollination is essential for the maintenance of wild and cultivated plant populations and biodiversity (Potts et al., 2010). The guild of pollinator is comprehensive of animals from very different taxa, including mammals (e.g., bats), birds (e.g., the hummingbirds) and reptiles (e.g., some lizards) (Proctor et al., 1996). However, insects are the most diffused responsible for pollination provisioning in natural systems, but also in the agricultural/urban contexts (IPBES, 2016).

Bees (Hymenoptera, Anthophila) are widely recognised as the most abundant group of pollinators. These insects are distributed worldwide with at least 20.000 species (Michener, 2000) and at least 785 of them are known to visit crops (Kleijn et al., 2015). Managed species, such as the honeybees (e.g., *Apis mellifera* L., 1758,

and *A. cerana* F., 1793), are often considered by the media as the most important pollinators (Smith & Saunders, 2016); however, evidence proves that high diversity of pollinator species is necessary to promote an efficient pollination service (Garibaldi et al., 2013). Indeed, high species diversity also means higher diversity in body size, shape, flight ability and other traits recognised as “functional”, but it also means higher spatial and phenological complementarity (Kendall et al., 2019). This functional trait diversity and complementarity is necessary to ensure that all the different flowers occurring in a community, with heterogeneous morphology, shapes, and phenology can be effectively visited by the community of pollinators (Fründ et al., 2013). The success of bees as pollinators largely matches with this required functional diversity. Bees are heterogeneous in body size and have specific coevolutionary traits with flowers (Anderson & Johnson, 2008). While several bee species are polylectic and forage on a wide variety of plants, many others are specialized in visiting a host flower they depend totally on (Cane & Sipes, 2006). Similarly, plants may depend on specific bee traits. For example, several plant species release pollen only in presence of specific vibration emitted by insects (De Luca & Vallejo-Marin, 2013). This is the case of bumblebees (*Bombus* spp.) that are among the few effective pollinators of tomato in greenhouses, due to a process known as the “Buzz pollination” (Ahmad et al., 2015). Moreover, bees can transport a large number of pollen grains due to their hairy bodies, and several bee families have specific body structures to collect pollen, as *corbiculae* or *scopae* (Engel et al., 2020).

Other insect orders as Coleoptera, Lepidoptera, but also other Hymenoptera, received less attention than bees but largely contribute to pollen dispersal service, and widely mediate crops pollination (Rader et al., 2016). Flies (order Diptera) and especially those belonging to the Syrphidae family are one of the major groups of non-bee flower visiting insects, and they are among the most important pollinators (Forup et al. 2008; Klecka et al., 2018). Their role is often neglected or underestimated, but hoverflies use to visit a comparable proportion of flowers than those visited by Hymenoptera, and are dominant pollinators in certain habitats (e.g., high altitudes, or arctic regions) (Kanstrup & Olesen, 2000).

The importance of pollinator insects for human wellbeing is mainly related to their agronomical importance. Lautenbach et al. (2012) estimated that the global value of pollination for food production is near to \$351 billion (USD)/year. About

75% of our global crops are pollinator dependent (Klein et al., 2007). Moreover, in the last fifty years agriculture pollination dependency, thus its vulnerability to pollination loss, significantly increased (Figure 1 - Potts et al., 2016 a).

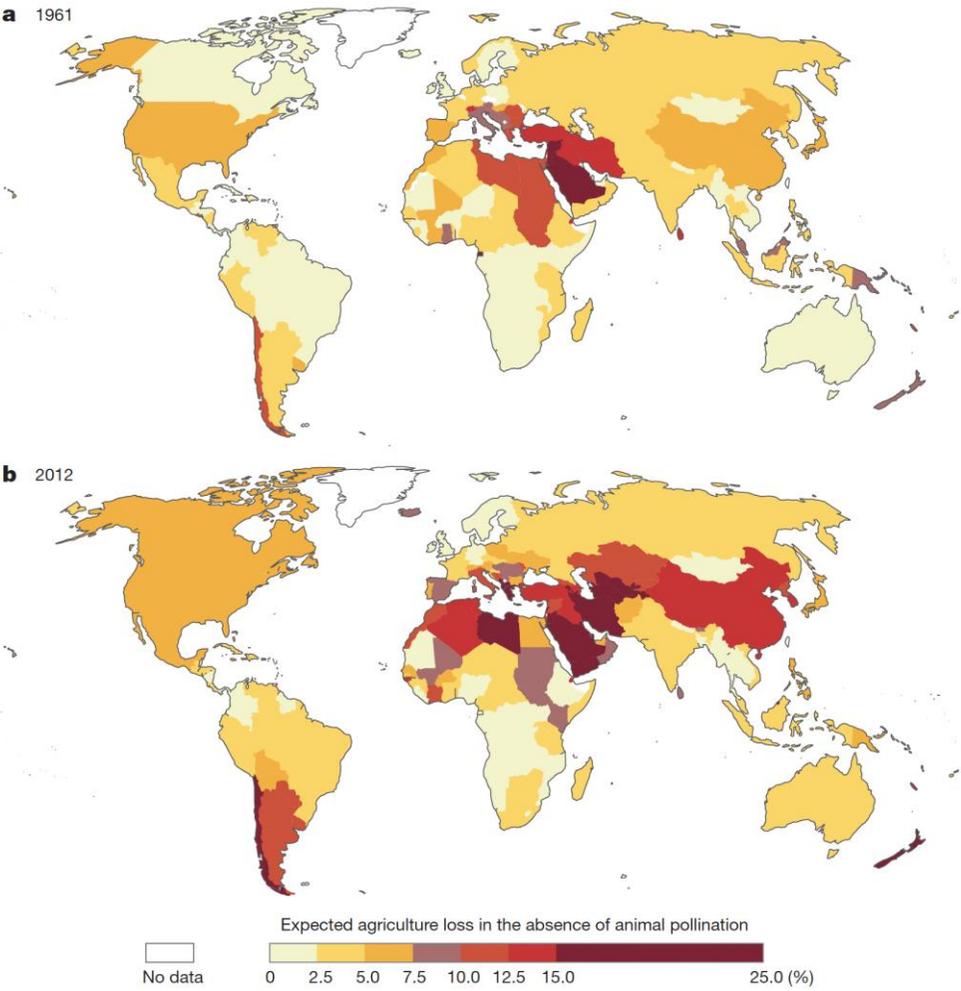


Figure 1: Pollination dependency of agriculture in a) 1961 and b) 2021, colours refer to the expected agricultural loss in absence of animal pollination, darker colours reflect higher vulnerability to pollination loss. Figure taken from Potts et al., 2016 a.

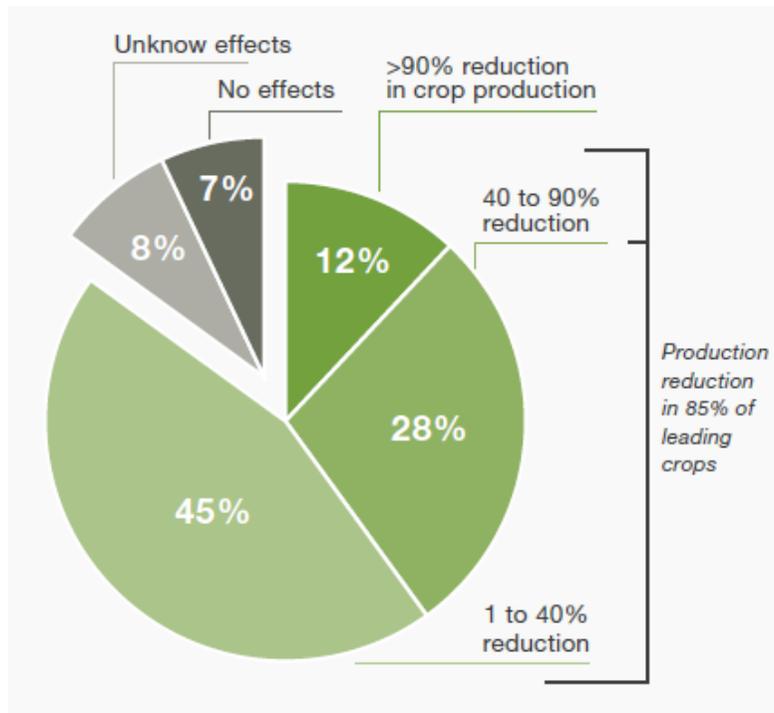


Figure 2: Dependence on animal pollination (percentage) for 107 leading global crops intended for direct human consumption. The plot is taken from IPBES (2016) and based on results from Klein et al., 2007.

Agricultural yield and quality of products are directly related to pollination for the 85% of the 107 crops intended for human consumption (see Figure 2; Klein et al., 2007). It is well proved that a higher pollinator species richness and abundance increase pollination success (Garibaldi et al., 2013) and that these results can be achieved by adopting several practices of land use management (e.g., planting flower strips) that lead more stable and increased agricultural yields (Garibaldi et al., 2014). Interestingly, these benefits even involve autogamous plants, as already proved in the case of valuable cash crops such as cotton, sesame, and coffee (Classen et al., 2014; Stein et al., 2017).

However, not only yields but also the nutritional value of agricultural products is improved by animal pollination. The pollination ecosystem service, indeed, is also implied in the production of most of the crops known as sources of micronutrients, as vitamin A and C, fluoride, and folic acid (Smith et al., 2015). Therefore, pollination is directly linked with the quality of human diet and the micronutrient deficiency

known as 'hidden hunger' (van der Sluijs & Vaage 2016). Indeed, Chaplin-Kramer and colleagues (2014) found out the occurrence of a spatial-overlap between the pollination dependency, thus the distribution of pollinator-dependent crops, and the micronutrient deficiency related pathologies (i.e., non-communicable diseases). Together, these aspects related to the interplay between pollinators and different features of agricultural production, clearly highlight the connection existing among pollination, food security and human wellbeing.

However, the importance of pollinator insects goes far behind their role in crop pollination and maintenance of biodiversity. Bees are natural allies of considerable cultural value for humans since immemorial time. For example, honey hunting scenes on rock paintings can be dated back to 6000 BC or earlier (Jones, 2009). Moreover, bees are part of religious or mythological scriptures (Potts et al., 2016 b), and nowadays, beekeeping is usually referred to as way to 'connect with nature' (Stange et al., 2018). Furthermore, bees have traditionally been used as bioindicators for the occurrence of contaminants (Bargańska et al., 2016). Bee products (e.g., honey, wax, venom) also provide unvaluable items for traditional and modern medicine (Kocot et al., 2018). A further confirmation of their importance for human wellbeing is that bees have been found to potentially contribute to the achievement of 15 of the 17 Sustainable Development Goals (SDGs) of the United Nations, and about 30 SDGs targets (Patel et al., 2021; Figure 3), as they are implicated in several social, cultural, and economic issues.

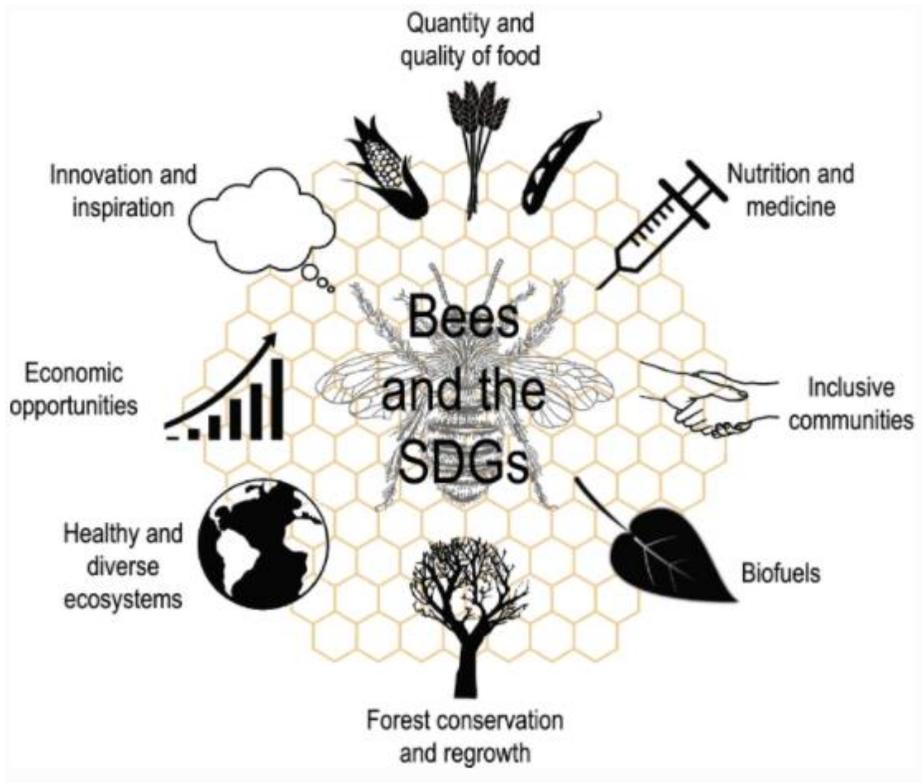


Figure 3: General themes for which bees contribute to sustainable development target. The Image is taken from Patel et al., 2021.

The recognition and the perception of the importance of bees is rapidly increasing in the population (Dicks et al., 2021). The global decline of the pollinator insects is attracting the global attention towards the need to preserve pollinators and consequently all the multiple benefits they provide to humans (Potts et al., 2016 a). In the light of the “One health” concept, the health of people is strictly connected to the health of environment in all its biotic and abiotic parts (Galimberti et al., 2020), thus the safeguarding of pollinator insects’ wellbeing is mandatory to maintain a healthier lifestyle of citizens.

2. THE GLOBAL DECLINE OF POLLINATOR INSECTS

Despite the multiple benefits that pollinator insects provide for human health and wellbeing, several evidence proves that in the last fifty years this animal group is facing a worrying global decline (Potts et al., 2010; Vanbergen, 2013, Goulson et al., 2015; Dicks et al., 2021). A significant decrease in wild bee abundance and diversity has been reported worldwide; however, for hoverflies (Syrphidae), butterflies, moths, and other pollinator insects this issue is more localized (Ollerton, 2017). Several studies also suggest that domestic honeybees are facing the same trend in Europe and North America, although the number of their colonies is increasing in other countries due to the spread of apiculture activities (Moritz & Erler, 2016). The ongoing decline is globally drawing both public and policy maker attention, and several actions have been taken to face this problem (e.g., EU Pollinator Initiative, 2020), since this rapid biodiversity loss is threatening ecosystem functions and food security (Aizen et al., 2009; Vanbergen, 2013; Goulson et al., 2015).

The growing human population is rapidly changing the worlds ecosystems, and several anthropogenic related pressures are supposed to largely drive this pollinator biodiversity loss (Potts et al., 2010; Vanbergen, 2013).

Climate change is one of the main consequences of human activities expansion, mainly occurring as a direct consequence of the increased emission of greenhouse gasses in the last hundred years (Heshmati, 2020). This phenomenon is affecting the distribution of both plant and pollinator species and is shaping their phenological cycles (Bartomeus et al., 2013; Forrest, 2015; Gómez-Ruiz & Lacher, 2019). The distribution of most bumblebee species, for example, has been reduced by the increasing temperatures (Kerr et al., 2015). Changes in species phenology in response to climate change are supposed to disrupt the synchrony between plants and their pollinators (Bartomeus et al., 2013). While polylectic pollinators are expected to easily adapt to these changes in the available floral resources, detrimental consequences are supposed to mainly affect specialist pollinators (Vanbergen, 2013). Shifts in the phenological overlap of plants and their pollinators may also inevitably affect the pollination of several crops (Settele et al., 2016) with detrimental consequences involving food security issues.

The increasing human population is also leading to an intensification of agricultural activities, reflected by an increased use of agrochemicals (Majeed,

2018). Moreover, agrochemicals (e.g., insecticides, fungicides, herbicides etc.) are not only widely employed in agriculture for plant protection but also are involved in public health related issues for the control of disease vectors (Sarwar, 2016). These chemicals are usually designed to have a minimum impact on bees, also due to specific application guidelines. However, these often do not consider the physiology, phenology, and the behaviour of most wild pollinators, that in turn may be deeply affected by them (Vanbergen, 2013). Agrochemicals may directly affect pollinators through acute and deadly intoxication events (Centrella et al., 2020). However, insects are mostly exposed to low environmental concentration of pesticides, that results in chronic sublethal effects that threaten the long-term survival of pollinator populations, affecting insects' behavioural and physiological aspects (Potts et al., 2010). Furthermore, in many countries agricultural plant protection also relies on genetically modified organisms (GMOs), that are usually crops with added function for herbicide tolerance or herbivory toxicity. The transgenes of these plants are also expressed in their pollen and nectar and their ingestion is supposed to affect physiology and survival of pollinators (Sanvido et al., 2007).

The spread of exotic species is another major threat for pollinator biodiversity conservation that occurs accidentally (e.g., goods cargo) or deliberately (e.g., exchange of ornamental or horticultural species) (Vanbergen et al., 2018). The spread of alien predators can easily reduce native population or shape the composition of pollinator communities. An example is the recent introduction of the yellow-legged hornet (*Vespa velutina*) from Asia, a bee predator that is threatening the European honeybees (Monceau et al., 2014). The introduction of alien species may also lead to deep alteration of plant-pollinator interaction patterns (Bartomeus et al., 2008). Alien pollinators can compete for the same resources of indigenous insects pushing them to extinction (competitive exclusion) as in the case of the extirpation of the Patagonian giant bumblebee *Bombus dahlbomii* from its original range following the establishment of the European species *B. terrestris* and *B. ruderatus* (Morales et al., 2013). At the same time, invasive plants can compete with the local ones by reducing their reproductive success (Morales & Traveset, 2009) or modifying the nutritional balancing of pollinators' diet (Vanbergen et al., 2018).

The diffusion of invasive species also leads to the diffusion of pollinator

diseases and pathogens. A well-known example is the spread of *Varroa destructor*, an Asian native mite originally diffused only in *A. cerana* colonies that is globally distributed nowadays. The consequences of this infection are deleterious as while it is harmless in *A. cerana*, it is detrimental and deadly for *A. mellifera* colonies (Rosenkranz et al., 2010). Moreover, the spread of this mite is driving the diffusion of other pathogens, for example the Deformed-wing virus (Wilfert et al., 2016). More in general, wild pollinators suffer from a wide range of fungal, bacterial viral and protozoan parasites and the risk of their global diffusion is exacerbated by commercial trades and the consequent biological invasions (Potts et al., 2016 a).

The increased pollution caused by industrial activities, urbanization, and agricultural intensification is another issue of global concern that may be involved in pollinators decline. For example, air pollutants, as volatile organic compounds, alter plant-pollinator interactions by messing with chemical communication mechanism (Jürgens & Bischoff, 2017). Similarly, soil contaminants shape mutualistic interactions leading to shorter flower visitations (Sivakoff, & Gardiner, 2017). However, the effects of pollutants on pollinators are less investigated in the current literature (Wenzel et al., 2020), and only few studies examined the response to air pollutants in wild systems (Thimmegowda et al., 2020).

All these stressors could act simultaneously and synergistically on pollinator communities (Potts et al., 2016 a). For instance, climate change possibly affects the availability of floral resources by altering their phenology (Bartomeus et al., 2013), in turn this can increase the vulnerability of pollinators to parasites, since infections usually increase the demand for specific nutrients (e.g., the infection by *Nosema ceranae* increases the daily carbohydrate intake) (Vanbergen, 2013).

Despite the well-known importance of all the mentioned stressors in leading the pollinators decline, landscape composition, configuration and land use intensification are globally recognised as the most important drivers of this biodiversity loss (Potts et al., 2010; Dicks et al 2021).

3. THE EFFECTS OF LAND USE INTENSIFICATION ON POLLINATOR DIVERSITY AND INTERACTIONS

An ongoing process of changes in natural ecosystems is occurring worldwide following the human population growing rate (Foley et al., 2005; Pecl et al., 2017; Díaz et al., 2019). The increasing demand for food and living spaces is leading to a land use intensification process through the conversion of natural areas into urban and agricultural landscapes. Both pollinator biodiversity and ecological functioning are widely affected by these changes (Wenzel et al., 2020; Fisogni et al., 2020), due to modifications in landscape composition and configuration (e.g., fragmentation, isolation). These processes lead to habitat loss, degradation, and fragmentation and are mainly driven by the land use intensification related to urbanization and agricultural intensification (Harrison & Winfree, 2015; Kovács-Hostyánszki et al., 2017). Although the response of pollinator communities to these factors also relies on local environmental conditions (e.g., floral resource availability, microclimate condition, etc.) (Vrdoljak et al., 2016; Prado et al., 2021), some general trends exist about how pollinators react to these alterations.

Urbanization is a worldwide and increasing phenomenon. The United Nations suggest, for example, that the 68% of the world population will live in urban areas by 2050 (UN – Department of Economic and Social Affairs, 2018). The spread of urban landscapes is inevitably linked with the increasing amount of land surfaces covered by concrete (usually named as impervious surfaces), because of the higher abundance of streets and infrastructures. Urbanization not only affects landscape composition by increasing the proportion of impervious surfaces, but also affects the spatial configuration (e.g., fragmentation) of the remaining green patches (Harrison & Winfree, 2015). A recent review (Ayers & Rehan, 2021) suggested that the urban landscape acts as a filter for functional traits of pollinator communities, specifically through habitat loss and fragmentation, (Figure 4).

These phenomena reduce the availability of soil nesting sites, and force pollinators to longer foraging trips to reach flower resources that could be scattered on the territory (Wenzel et al., 2020). Therefore, in urban contexts, the cavity nesting bees will be favoured instead of the ground-nesting ones because of the low availability of suitable bare soil (Ayers & Rehan, 2021). Moreover, smaller bees should be disadvantaged by the lower connection between green patches because smaller insects are usually less able to disperse (Greenleaf et al., 2007).

Furthermore, changes in the landscape composition, specifically the higher concrete abundance, is also related to the occurrence of peculiar local conditions.

Among these, the higher urban temperature, a phenomenon known as 'heat island effect', affects pollinators in multiple ways. First, by shifting plants and pollinator phenological overlap (Bartomeus et al., 2013), secondly by favouring the more tolerant species (Hamblin et al., 2017). Moreover, pollinator insects are also expected to adapt their body size to cope with the higher temperatures of urban landscapes, since smaller individuals are less exposed to the risk of overheating while foraging (Gérard et al., 2018).

Landscape features and the resulting local climatic conditions also affect other biotic features such as the structure and the composition of urban vegetation, with invasive and ornamental plants more commonly found in cities (Harrison et al., 2016), and through species phenological shifts (Luo et al; 2007). These changes in plant community further supports the idea that smaller and polylectic pollinator insects, that efficiently exploit all the available resources, should be favoured in urban habitats. (Wenzel et al., 2020).

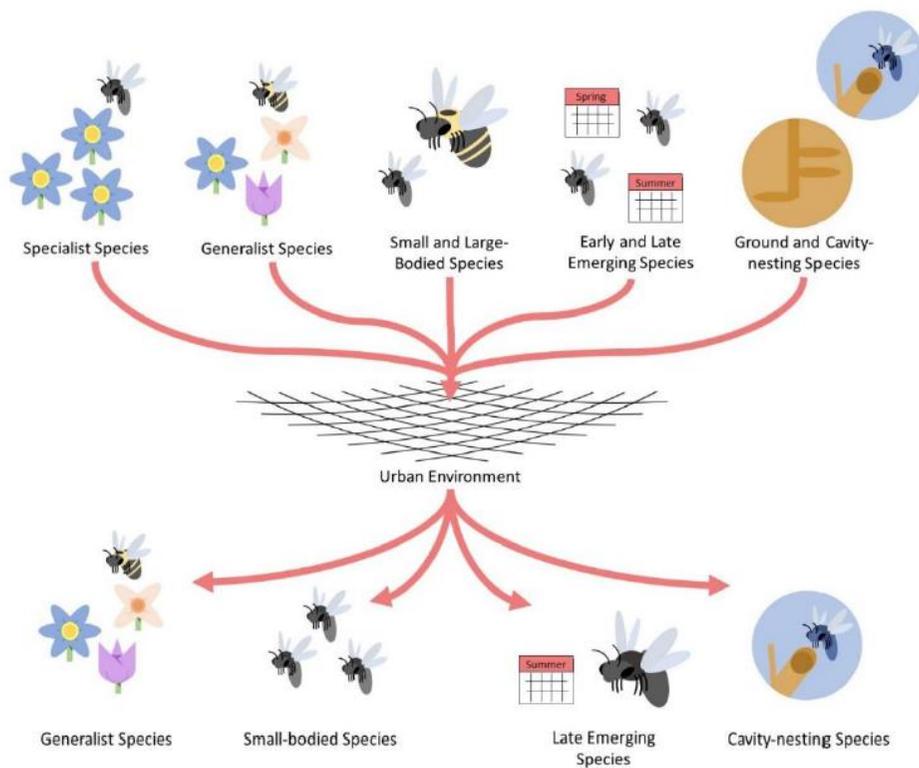


Figure 4: The diagram summarizes the filter effect of the urban environment (represented by the net). The landscape composition, configuration, and specific local conditions (e.g., microclimate, vegetation) related to the urban environment influence the structure of the bee communities. Generalist, smaller, and cavity nesting species are favoured in urban landscapes. The image is taken by Ayers and Rehan (2021)

By filtering pollinators with specific functional traits, the urban landscape also represents a refuge for certain pollinator species that well adapt to this anthropic condition (Theodorou et al., 2020). Despite the high rate of habitat loss and fragmentation, urban areas were found to host higher pollinators biodiversity than their rural counterparts, especially with intermediate proportion of impervious surfaces (i.e., lower than 50%) and when green spaces for nesting and foraging are available (Wenzel et al., 2020).

Urbanization does not only affect the functional biodiversity of pollinator insects, but also their mutualistic interactions with plants (Harrison & Winfree, 2015). Lower trophic specialization commonly occurs in cities when compared with rural or urban habitats, possibly because of the previously mentioned increased abundance of generalist species. Changes in foraging behaviour (e.g., time spent on flowers or foraging distance) could also result from a condition of higher

fragmentation of resource patches and are adopted to prevent insects from spending energy for traveling among remote patches (Harrison & Winfree, 2015). Despite these changes in plant-pollinator interactions, a few studies investigated how urbanization affects the pollination service, finding no negative effects on native plants or crops (as reviewed by Wenzel et al., 2020).

The other face of the spread of human population is represented by the spread and intensification of agricultural activities that only in Europe occupy up to the 47% of the territory (EEA, 2010).

Agricultural intensification affects landscape composition and configuration and similarly to urbanization, it leads to habitat loss, degradation, and fragmentation (José-María et al., 2011; González-Robles et al., 2020), with severe consequences for pollinators. Specifically, agricultural intensification (e.g., creation of large monocultures) lead to simplified landscapes and reduced suitable habitat availability (Foley et al., 2005) with detrimental consequences for the pollinator diversity, their interaction with plants, and thus the efficiency of the pollination ecosystem service (Steffan-Dewenter & Westphal, 2008; Mogren et al., 2016; Rollin et al., 2016). While monocultures largely dominate agricultural landscapes, evidences proves that increasing the heterogeneity of crops fields could promote biodiversity without affecting yield (Hass et al., 2018) and that not only heterogeneity, but also the spatial arrangement of high-quality habitats is an important trait to promote biodiversity (Steffan-Dewenter & Westphal, 2008).

Moreover, the fields distance from natural or semi natural habitats is known to improve the species richness in agricultural landscapes and consequently the provided pollination service (Steffan-Dewenter, 2003; Garibaldi et al., 2011). Semi natural habitats are important sources of resources in landscapes largely characterized by crop mass flowering events. These provide valuable but highly unstable food resources for pollinators, that only occur for limited periods of the pollinator phenology (Classen et al., 2014). Therefore, the proximity to semi natural areas could prevent pollinators from suffering resource shortage.

At last, agricultural intensification not only affects pollinators through landscape modification, but also at the local scale through the extensive application of fertilizers and pesticides that not only kills pollinators directly, but especially reduce the amount of available flower resources and their distribution (Steffan-Dewenter & Westphal, 2008; Mogren et al., 2016, Cole et al., 2017) with detrimental consequences for pollinator survival.

Overall, urbanization and agricultural intensification are characterized by peculiar local conditions able to shape pollinator communities features that, however, are

mainly impacted by changes in landscape composition and configuration.

The alteration of landscape composition leads to habitat loss and degradation. This reduces plant and pollinator diversity, since the more specialized insects and plants could disappear as their suitable habitat is progressively lost (Fisogni et al., 2020). At the same time, all the plant and pollinator species are expected to suffer from the decrease in habitat quality induced by landscape composition changes (Wenzel et al., 2020).

Alteration in the landscape configuration could also affect pollinators and plants through various and complex mechanisms, and general decrease in species richness is usually associated with increasing fragmentation of the seminatural patches (Xiao et al., 2016). The consequences of landscape composition and configuration changes also impact the pollination service that insects provide, and thus in the reproductive success of plants (Montero-Castaño & Vila, 2012). This close interconnection between land use related drivers of pollinator declines, their effect on plant and pollinator communities, and the consequent efficiency of the pollination service are summarized in Figure 5.

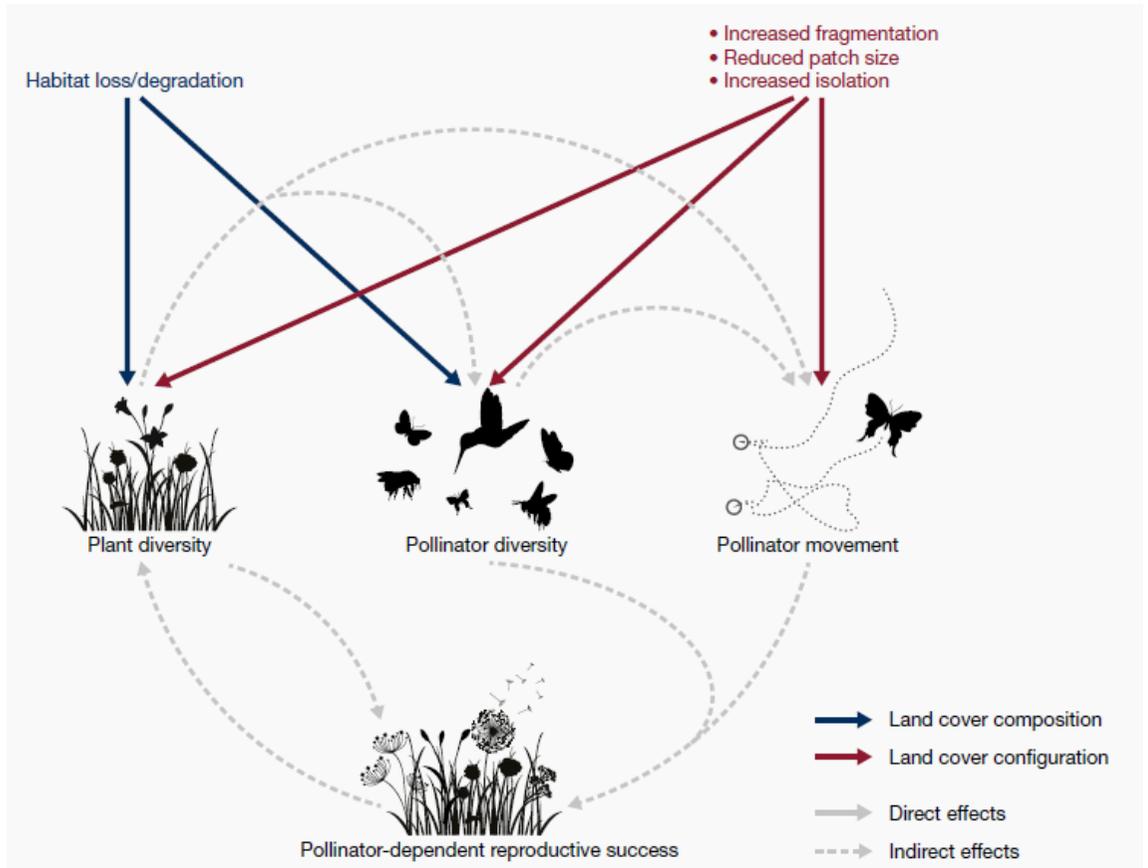


Figure 5: The diagram shows the direct and indirect effect of habitat loss and degradation resulting from changes in landscape composition, and the effects of habitat fragmentation resulting from changes in landscape configuration. The image is taken from IPBES, 2016.

Overall, the rate of land use intensification, both in response to urbanization and agricultural land expansion, is proceeding rapidly worldwide. These phenomena are especially occurring in developing countries, which usually host high biodiversity, such as Sub-Saharan Africa and Asia, where the urbanization is proceeding at the highest rate on Earth and is expected to continuously increase in the next future (Sulemana et al., 2019). The whole scientific community should act to find suitable solutions to face the pollinator insects decline. This requires the need to improve our comprehension of drivers and consequences of biodiversity loss, also involving those countries more affected by the alteration of ecological systems.

4. ASSESSING THE EFFECTS OF LAND USE AND THE RELATED STRESSORS ON POLLINATORS: MULTILEVEL INVESTIGATION AND OPEN ISSUES

A global effort in scientific research is required to improve our comprehension on how the stressors related to land use intensification and their synergic combinations are affecting the pollinator communities. This is necessary not only to better understand the theoretical ecology-based aspects of such alterations, but also to design suitable contingency strategies to contrast the biodiversity decline of pollinator insects. For example, a complete understanding of the processes driving pollinators diversity, fitness and interactions could permit the development of effective nature-based solutions (e.g., promoting hedgerows, or flower strips plantation) that could be incorporated into policy guidelines for the territory management.

Despite this global priority, most of the pollinator-related studies comes from North America and Europe, while a significant knowledge gap exists for the rest of the world (Figure 6), especially concerning Sub-Saharan Africa, most part of Asia and their insular systems (Timberlake and Morgan, 2018). While in these countries the research on pollinators diversity and ecology is largely neglected, the agricultural dependency from pollination has increased with higher rates in the last fifty years (Potts et al., 2016 a - Figure 1). These areas, in turn, also result more vulnerable to the consequences of pollinator loss if compared to the rest of the world.

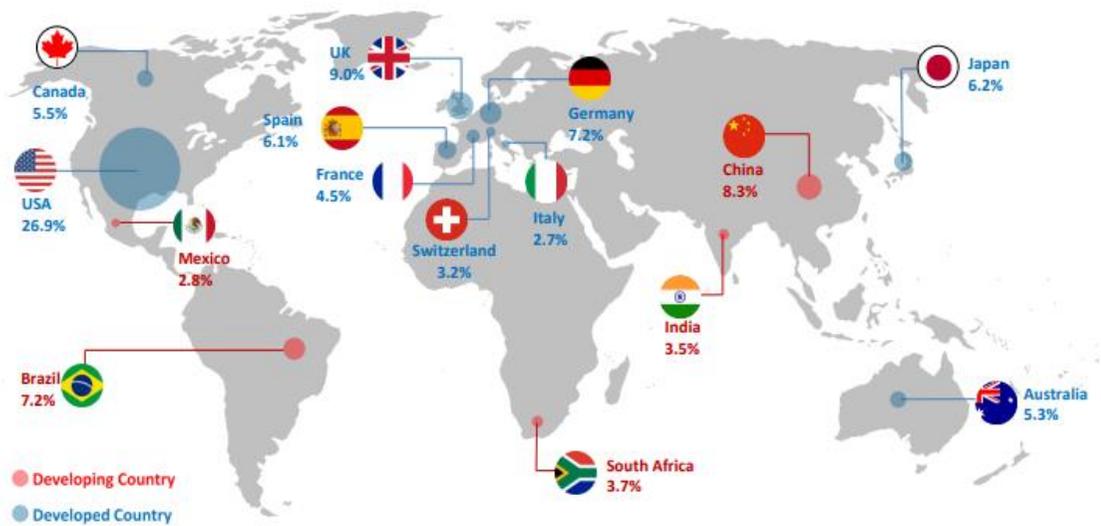


Figure 6: The map is taken from Timberlake and Morgan (2018) and shows the 15 top countries that between 1998 and 2018 contributed to pollination research papers. The map highlights a significant data gap for most of the developing countries.

Other gaps exist in our comprehension of how pollinators are affected by stressors directly or indirectly related to land use features.

On one hand, the effects of abiotic local scale features occurring as consequence of increased urbanization rate (e.g., the urban heat islands effect, the environmental pollutants or changes involving climatic features as humidity) have been rarely investigated on pollinator communities (Wenzel et al., 2020). Conversely, the role of biotic factors (e.g., availability of floral resources) in shaping the features of pollinator communities (e.g., pollinator richness or abundance) has been more investigated (Montero-Castaño & Vila, 2012). However, the synergic relation between these local biotic features with landscape characteristics (e.g., composition and configuration) must be further investigated. Moreover, qualitative alterations of local biotic features such as variations in the nutritional profiles of floral resources in response to land use intensification and the related stressors may be expected (Vaudo et al., 2015; Vaudo et al., 2018) and should be further investigated with the same priority.

Lastly, contrasting responses to landscape composition and configuration changes were previously observed in literature (e.g., low degrees of habitat alteration increase pollinator richness). However, it is still necessary to clarify which local biotic/abiotic or landscape features are the main responsible for these contrasting results.

The outcomes of these investigations could be useful for developing best practices in land use management and should be incorporated in policy actions aimed at safeguarding pollinators. To successfully achieve these goals, a more integrative approach to pollinator/pollination studies that consider multiple aspects of pollinator communities at the same time, could be more informative. Indeed, several studies related to pollinators focus on the effects of stressors (e.g., land use alteration) on biodiversity, but they are not always suitable to highlight impacts on individuals of certain species, or on community behaviour.

In a complex insect community, some species can be more important than others, for example because more abundant or more efficient in maintaining the stability of pollination service. Thus, investigating how individuals of certain species are influenced by stressors related to landscape features, for instance in terms of survival or development of specific adaptations, can provide suitable information for the conservation of these focal species.

Not only biodiversity, but also the interactions occurring among species are important descriptors of complex communities. Indeed, the study of mutualistic interactions between pollinators and plants offers a broader panel of information than a simple biodiversity assessment (Albrecht et al., 2012; Pollock et al., 2020). Investigations involving the study of interactions are useful to describe and predict both species and community level responses to environmental stressors. This strategy permits to evaluate the stability of plant-pollinator interactions (Peralta et al., 2020), determine how this stability is shaped by landscape features (Redhead et al., 2018), and find the most important species for the maintenance of pollination network stability (Biella et al., 2019 b).

Multidisciplinary investigation approaches, addressing different levels of complexity (i.e., from single species to community investigations) of biodiversity and interactions, could be even more informative when coupled with measures of pollination success (i.e., fruit production or counts of pollen tubes germination on stigmas). This allows to directly link the variation in species or community features, possibly induced by land use intensification or other related stressors, to variation on the efficiency of pollination.

Other aspects, such as the nutritional quality of floral resources, and their variation in response to environmental stressors should be considered along with the previously mentioned aspects related to species, community, and functional

features. Nutritional aspects, in fact, can impact insect morphology (Filipiak, 2019), mutualistic interactions (Nepi et al., 2018; Latty et al., 2020), and consequently may affect pollination efficiency as well.

A research approach involving these multiple investigation levels could be highly informative but challenging and would require a considerable investment in terms of time and resources for field, laboratory, and computational activities. However, technological advances in the field of molecular biology, such as the DNA barcoding and DNA metabarcoding, may simplify the way this multilevel investigation is achieved, for example by speeding the process of species and interactions identification (Bell et al., 2017). Moreover, variables related to landscape composition, configuration, and other environmental conditions such as the temperature or precipitation, can be successfully recovered by available land use maps coming from national or international resources (e.g., CORINE Land Cover (Heymann et al., 1994)) or by remote sensing approaches (e.g., MODIS, further details in Chapter 7 and 8.5).

In the research works presented in this PhD thesis, the effects of landscape composition, configuration, and of the related biotic and abiotic stressors have been investigated considering multiple levels of investigation, to favour a more comprehensive understanding of the effects of these drivers on pollinators communities, possibly offering suitable solutions to safeguard pollination.

5. AIM OF THE PHD PROJECT & EXPERIMENTAL DESIGN

The main scope of this PhD thesis was to provide advances in the comprehension of the effects that stressors related to land use intensification have on multiple aspects of pollinator communities, particularly, species functional traits, biodiversity, interactions of pollinator communities with plants, and pollination efficiency. Together, these aspects have been addressed in the study cases reported in this thesis both to solve ecological questions and to provide suitable policy suggestions for pollinators conservation. At the same time, this PhD work aimed at improving the available knowledge about pollinator insects in some countries largely neglected by pollinator-related research.

The general aims of this thesis have been achieved through the following intermediate goals.

- Assessment of the impact of landscape composition and local biotic variables (e.g., proportion of impervious surfaces and flower abundance) on the biodiversity and interactions with flowering plants of pollinator insects.
- Investigation of the impact of green habitats fragmentation on pollinator insects' biodiversity, plant-pollinator interactions, and pollination efficiency of wild plants.
- Assessments of the effects of landscape composition, configuration and microclimate conditions induced by urbanization on the diversity of pollen foraged by insects, and on the sugar content of nectar from spontaneous flowers.
- Investigation of the impact of abiotic and biotic stressors related to urbanization on species functional traits (e.g., wing size and shape asymmetry).

These intermediate aims have been addressed in detail (separately or combined) in the case studied presented in the next chapters. The overall experimental design of the PhD project is depicted in Figure 7.

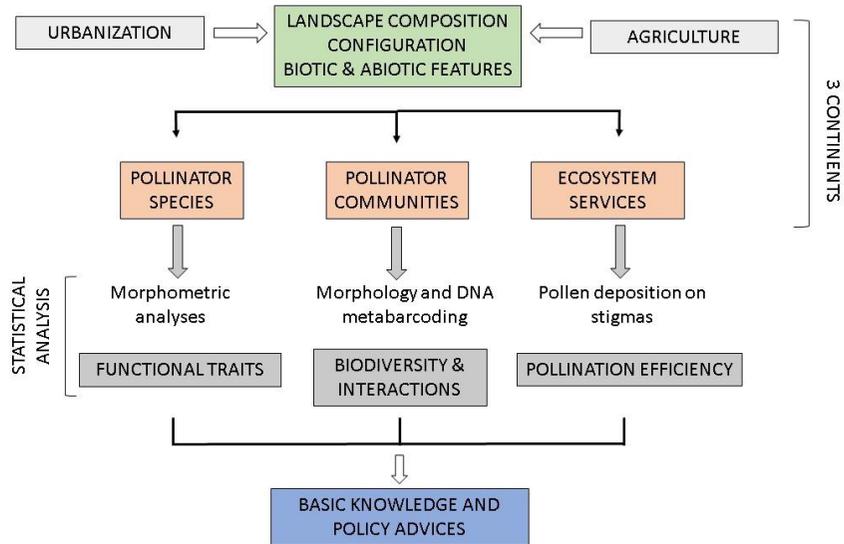


Figure 7: Conceptual map regarding the general aim of this PhD project. The effects of landscape composition, configuration, and the related changes in biotic and abiotic environmental features, on multiple aspects of pollinator communities have been investigated. These environmental variables have been quantified as consequence of urbanization and agricultural land expansion. By integrating different methodological approaches, the effects of the mentioned stressors were assessed on species functional traits, community biodiversity and interactions, and pollination efficiency. Three continents (i.e., Europe, Africa, and Asia) were involved in this research. The outputs of this work were valorised in the scientific publications listed in this thesis, that not only provide an advance in terms of basic ecological knowledge, but also advices for the development of suitable management policy actions.

6. STUDY CASES

Both the general and the intermediate aims of this PhD project have been achieved through the study cases reported in Chapter 8 and are briefly overviewed here. The first study (Chapter 8.1) has been conducted on the smallholder farming system of Arusha, Tanzania, Africa. The impacts of landscape composition and of local biotic variables (e.g., floral resources availability) has been investigated on pollinator species richness and on their patterns of interaction with plants. Since an efficient pollination service is directly related to agricultural yield and product quality (Bommarco et al., 2012), this study aimed at identifying the land use management strategies able to promote a sustainable agriculture development based on diverse and efficient pollinator insect communities. This work has been published in the journal “Agriculture, Ecosystems and Environment” in June 2021. It has been supported by the SASS project (Sustainable Agri-Food System Strategies) founded by the Italian Ministry of University and Research (MIUR) that aimed at improving the sustainability of food systems in Sub-Saharan Africa.

The second study case (Chapter 8.2) focused on the effect of green habitat fragmentation on pollinator richness, on the interaction with plants at the community and intraspecific level, and on the pollination efficiency. This study took place in islands of the Maldives archipelago, a context that offers ideal experimental conditions given their isolation and simplified ecological communities. Moreover, these islands are characterized by a gradient of green habitat fragmentation that permits to clearly investigate the effects of different stressors, related to landscape configuration, on pollinator community features and functionality. This study has been submitted to the journal “Molecular Ecology” and took place in the framework of an interdepartmental collaboration between MaRHe Center (Department of Environmental Science), and the Department of Biotechnology and Bioscience of the University of Milano-Bicocca.

In the third study case, the effects of landscape composition, and configuration on pollinator abundance, their foraging strategies, and the nutritional properties of flower resources have been investigated in the metropolitan area of Milan. Moreover, the role of microclimate conditions related to urban landscape in influencing the same pollinator and flower resource features has been analysed. This study case is available in preprint and has been submitted to the “Journal of

Applied Ecology” and performed in the framework of the project Pignoletto (Monitoring of the Territory and Precision Agriculture through Unmanned Aerial Vehicles) funded in the context of the R&D Hub call of Regione Lombardia.

The same projects also found the last study presented in Chapter 8, that focused on the effects of land use intensification, and the related abiotic/biotic stressors, on the functional dispersion traits of pollinator insects. Specifically, two model species of bees (*Bombus terrestris* and *B. pascuorum*) have been involved in this investigation that took place in the metropolitan area of Milan. This work is available in preprint has been submitted to the journal “Oecologia” and is currently undergoing its second round of revisions.

Finally, a methodological research study has also been included in this thesis. it provides useful advances in the application of molecular tools for the study of plant-pollinator interactions. Specifically, the effects of different strategies of filtering DNA metabarcoding raw data have been evaluated in terms of ecological interpretation of the plant-insect interactions. These results have been used as starting point to provide best practices for managing these molecular data. The results of this research have been published on the journal “Diversity” in September 2021.

The main field and laboratory methods used in these studies are explained in the following chapter.

7. METHODS

The study cases reported in the next chapters share several common methodological approaches related to the sampling activities, the covariate description, and the samples analytical processes.

Sampling site selection and covariates quantification

In the study cases included in this thesis (specifically those reported in Chapters 8.1, 8.2, 8.3, 8.5), the sampling sites for insects and/or flower resources have been selected to cover gradients of change in landscape features (e.g., composition and configuration) and/or of stressors directly related to landscape composition (e.g., temperature, precipitation).

The information required to visualize and quantify these gradients were recovered alternatively by creating land use maps through satellite images digitalization (as in Chapters 8.1 and 8.2) or by exploiting land use maps already available for the study sites (Chapters 8.3 and 8.5). Moreover, data resulting from remote sensing approaches have also been used for this purpose. Specifically, for the *de novo* creation of land use map, a buffer area has been drawn around each sampling plots starting from satellite images. The buffer size has been selected accordingly to the mean dispersion ability of the investigated pollinator insects (e.g., 500 – 1000 m of radius). Afterwards, in each buffer, polygons enclosing land use categories (e.g., impervious surfaces, green areas, agricultural plots) have been drawn. This allowed to directly quantify the land use composition around sampling sites, but also calculate configuration indices (e.g., Edge density). In the case of already existing land use maps for the investigated area, the same land use metrics have been calculated without the necessity of manually draw polygons for each land use category, but simply by isolating the interested buffer area around the sites from the whole map.

Other variables related to landscape features, such as the mean surface temperature that is largely influenced by the proportion of concrete cover (Xiao et al., 2007), have been recovered through a remote sensing approach. Land-surface temperatures maps, for example, were obtained from the data recorded by the MODIS sensor (moderate resolution imaging spectroradiometer, <https://modis.gsfc.nasa.gov/>).

The creation and management of land use map, quantification of landscape

composition and configuration features, as well as the recovery and processing of remote sensing data were performed in QGIS (QGIS Development Team, 2020). and are explained in detail in the study cases reported in the following chapters. Other local scale covariates, such as those related to vegetation and pollinator communities, have been directly evaluated through field observations.

Field sampling

Multiple approaches of field sampling were used to collect information on vegetation and pollinator community features.

Vegetation quadrats randomly places inside the sampling areas have been used to estimate parameters related to the availability of floral resources (e.g., species richness, flower abundance etc.). Specifically, this approach has been used in Chapters 8.1, 8.2, 8.5.

Pollinator insects have been collected using both active and passive collection methods. Specifically, pan traps (i.e., coloured bowls filled with water and a drop of soap and placed at the higher limits of the vegetation, Figure 8A), have been used to collect insects during fixed periods of 24 hours. In Chapter 8.3 sticky traps (Figure 8B), plastic boards covered with glue placed at 1 m height, have been used with the same exposure timing of pan traps. Both these methods allowed the estimation of pollinator species richness and/or abundance and examples of their application are reported in Chapters 8.1 and 8.3.

Insects were also collected by using entomological nets (Figure 8C). This approach has been used to catch the insects observed foraging on flowers along transects and during a fixed time. This second approach provides similar information than the previous one, but also permits to record information about plant-pollinator interactions also due to the molecular approach explained below. Insects collection have been performed only during good weather days, after having been collected the specimens were stored in 70% ethanol, or frozen until further analysis. Examples of the application of this sampling methods are provided in Chapters 8.1, 8.2, 8.3. 8.5.



Figure 8: Main sampling approaches used to collect insects in the works included in this PhD thesis. (A) Pan trap, coloured bowls filled with water and a drop of soap. (B) Sticky traps, plastic boards covered with glue. (C) Insects capture through entomological net.

Sample processing

Pollinator insect identification

In some of the case studies listed in this thesis (specifically in Chapters 8.1, 8.2) the taxonomic identification of the collected insects has been performed by integrating morphological and molecular identification approaches.

Morphological criteria have been used to identify insects at the species level, otherwise lower taxonomic ranks or morpho-species assessment were adopted. At the same time, a DNA barcoding approach has been used to confirm or to improve the taxonomic accuracy obtained through morphology.

The DNA barcoding is a molecular approach that exploits genetic makers, known as DNA barcode regions, to identify unknown samples by comparing the nucleotide sequence of these regions with known reference entries deposited in public repositories (e.g., BOLD Systems and NCBI-GenBank) (Hebert et al., 2003). The DNA barcode regions should be characterized by high interspecific and low intraspecific diversity and should be flanked by conserved sites for the development of universal PCR primers (Kress, & Erickson, 2008). The standard DNA barcode region for the identification of metazoans (including many pollinator insects) is the 5'-end of the COI mtDNA gene (650bp). In the case of the research presented in this thesis, the amplification primer HCO2198 and LCO1490 (Folmer et al., 1994) have been used for insect identification.

Plant-pollinator interactions analysis

To rapidly obtain information about plant-pollinator interactions an approach based on the analysis of the pollen grains carried on the body of insects have been used (specifically in Chapters 8.1, 8.2, and 8.3). Indeed, while visiting flowers for foraging, the insects actively or accidentally collect pollen grains, that can be used to unveil their foraging history. This approach is faster than the direct field observation of interactions and permits to retrieve information about the interactions that insects faced prior to their capture (Bosch et al., 2009). While morphological identification of pollen grains can be used to achieve this goal, a modern investigation approach based on DNA metabarcoding has been used in literature and in the study cases here presented. This approach has the advantage of being rapid and does not require high taxonomical knowledge (Bell et al., 2017). It is analogous to the DNA barcoding, but it is based on high throughput sequencing technologies, thus allowing the simultaneous identification of multiple species composing a single complex matrix (Deiner et al., 2017).

Briefly, after being washed from the body of insects, the mixtures of pollen grains have been processed through DNA extraction and amplification of the selected DNA barcode. After the DNA sequencing and comparison of the obtained DNA reads with the available reference sequences, the composition of each pollen mixture has been determined. This information has been used to create interaction matrices including the pollinator individuals and the species of pollen found on their bodies. Network indices have then been calculated from these matrices. These are numeric indices necessary to describe multiple features of plant-pollinator networks and make comparisons between contrasting conditions. A brief explanation on the significance of the main indices applied in the study cases included in thesis is reported in the following Table 1.

<i>Connectance</i>	Measure of the realised proportion of possible links. It is a measure of network complexity and specialization.
<i>H₂'</i>	Measure of the network specialization (both of pollinator and plants). It provides information on species functional redundancy and is useful for comparison among different interaction networks.
<i>Niche overlap</i>	Measure of the similarity in the interaction patterns among individuals or species of the same network level (pollinator or plant level). It provides information about competition or redundancy in the species functional role.
<i>Modularity</i>	Measure of the network structure, it evaluates how interactions are distributed into modules, where species within modules mostly interact with each other.
<i>Shannon Entropy</i>	Measure of the overall diversity in the interactions of a network. It provides information about the complexity of interactions.
<i>Degree</i>	Measure of the number of interactions observed for each pollinator (or plant) species/individual.
<i>Connectivity</i>	Measure of the role of a species (or individual) in connecting different parts (module) of the network. It can be used to evaluate the relative importance of species in contributing to network stability.

Table 1: Main network indices used in this PhD thesis and their significance, a detailed explanation of each index and further details on their calculation are included in the study cases reported in Chapter 8.

Figure 9 describes the main phases of this pollen DNA metabarcoding approach used to describe plant- pollinator interactions.

In all the study cases reported in this thesis, the nuDNA ITS2 DNA barcode region has been employed for pollen identification, because of its well-known species discrimination power (Chen et al., 2010). Since the availability of reference sequences is one of the main drawbacks for a successful pollen mixture identification (Biella et al., 2019 a), local reference databases, comprehensive of all the flowering species occurring at the study sites have been created. To do this, the ITS2 barcode region of all these flowering plants occurring at each sampling site, has been sequenced whenever the DNA metabarcoding approach has been applied.

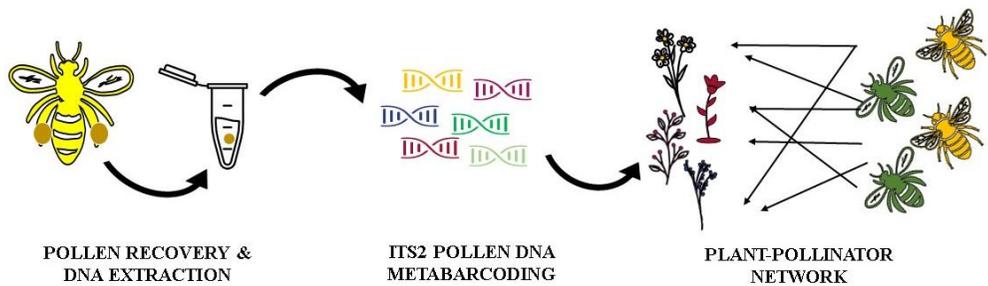


Figure 9: The diagram shows the approach used to retrieve information about plant-pollinator interactions through the DNA metabarcoding of the pollen mixtures carried on insects' bodies. Once isolated from the insects, pollen grains are processed through DNA extraction, amplification, sequencing, and consequently to the identification of the species composing the mixed pollen samples. This information is used to create matrices describing interaction between pollinator and plants, and thus to calculate network indices.

Geometric morphometrics analysis of functional traits

Changes in species morphological traits related to flight ability (e.g., body size and wing asymmetry) (Grilli et al., 2017; Soule et al., 2020) have been assessed through a geometric morphometrics approach. This is based on the analysis of landmark coordinates (Figure 10) and allows to quantify traits shape, size, and their variation (Cardini, 2013). Briefly, both side forewings of each individual belonging to the investigated pollinator species have been scanned and digitalized. After placing landmarks their coordinates have been used to estimate wing size and shapes. Afterward, these data have been used to estimate body size and wing size/shape asymmetry. Further details on this approach are provided in the study case reported in Chapter 8.5.

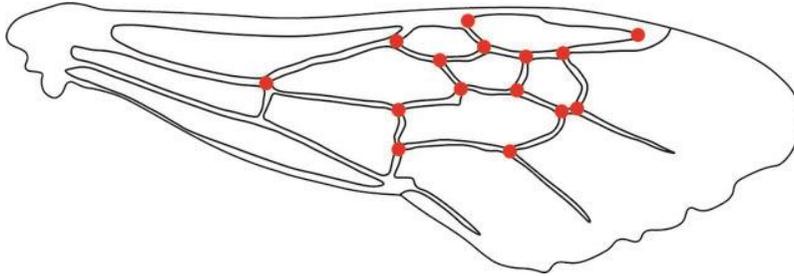


Figure 10: Example of landmark location on a right forewing of *B. terrestris*. The image refers to the landmark selected in the study case reported in Chapter 8.5

Pollination efficiency

The counts of germinated pollen tubes and pollen grains deposited on flower stigmas have been used to estimate variations in pollination effectiveness. The plant-pollinator interaction analysis provides indirect information about possible variation in the pollination efficiency (Arceo-Gómez et al., 2020). The count of germinated pollen tubes, instead, provides a direct measure of pollination effectiveness, and have been already applied for this purpose (Biella et al., 2019 b). Briefly, multiple stigmas of the main flowering species at a given site have been collected and preserved in a storage solution (66.5/33.5/2 EtOH/H₂O/CH₂O v/v/v). Once in the laboratory, these were softened using NaOH 1M, coloured with aniline blue, and mounted on microscopy slides. Pollen tubes, or pollen grains deposited on stigmas when tubes were not clearly visible, were visualized through fluorescence microscope and manually counted. Further details on this approach are provided in Chapter 8.2.

Statistical analysis

The data produced following the previously mentioned approaches, have been analysed through proper statistical pipelines. Regression analyses, based on linear and generalized linear models, have been mainly employed in the study cases included in this PhD thesis. Random effects have also been included in the models when required by the experimental conditions. Through this approach the trend of variation in pollinator community/individual features (e.g., species richness, network indices, pollination efficiency) in response to land use descriptors (e.g., proportion of impervious surfaces), or other variables related to them (e.g., temperatures, resource availability), have been successfully investigated. Detailed

explanations of the employed statistical approaches are provided in each study case reported in Chapter 8.

8. PUBLICATION PRODUCED IN THIS PhD

8.1 Tommasi, N., Biella, P., Guzzetti, L., Lasway, J. V., Njovu, H. K., Tapparo, A., ... & Galimberti, A. (2021). Impact of land use intensification and local features on plants and pollinators in Sub-Saharan smallholder farms. *Agriculture, Ecosystems & Environment*, 319, 107560.

In this work the first intermediate aim of the thesis has been achieved investigating pollinator biodiversity and their interaction with plants in the context of smallholder farms of Arusha, North Tanzania. The impact of land use composition and the role of local biotic variables has been determined integrating field sampling with laboratory activities of morphological and molecular identification and data analysis. I personally lead all these phases of this experimental work including writing and revision of the article.

Type of Article: Research article

IMPACT OF LAND USE INTENSIFICATION AND LOCAL FEATURES ON PLANTS AND POLLINATORS IN SUB-SAHARAN SMALLHOLDER FARMS

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Keywords: Bees, DNA metabarcoding, Ecosystem services, Hoverflies, Plant-pollinator interaction, Sustainability

ABSTRACT

Sub-Saharan African crop production largely relies on smallholder farms, located both in urban and agricultural landscapes. In this context, the investigation of plant and pollinator diversity and their interactions is of primary importance since both these factors are threatened by land use intensification and the consequent loss of natural habitats. In this study, we evaluated for the first time how plant and pollinator insect assemblages and interactions in Sub-Saharan farming conditions are shaped by land use intensification. To do that, we complemented biodiversity field surveys in Northern Tanzania with a modern DNA metabarcoding approach to characterize the foraged plants and thus built networks describing plant-pollinator interactions at the individual insect level. Moreover, we coupled this information with quantitative traits of landscape composition and floral availability surrounding each farm. We found that pollinator richness decreased with increasing impervious and agricultural cover in the landscape, whereas the flower density at each farm correlated with pollinator richness. The intensification of agricultural land use and urbanization correlated with a higher foraging niche overlap among pollinators due to convergence of individuals' flower visiting strategies. Furthermore, within farms, the higher availability of floral resources drove lower niche overlap among individuals, while a greater flower visitors abundance shaped higher generalization at the networks level (H2'), possibly due to increased competition. These mechanistic understandings leading to individuals' foraging niche overlap and generalism at the network level, could imply stability of interactions and of the pollination ecosystem service. Our integrative survey proved that plant-pollinator systems are largely affected by land use intensification and by local factors in smallholder farms of Sub-Saharan Africa. Thus, policies promoting nature-based solutions, among which the introduction of more pollinator-friendly practices by smallholder farmers, could be effective in mitigating the intensification of both urban and rural landscapes in this region, as well as in similar Sub-Saharan contexts.

INTRODUCTION

Anthropogenic land use conversion and intensification are among the major drivers of landscape changes and habitat loss in natural and semi-natural contexts (Foley et al., 2005; Graitson et al., 2020). At the global scale, the growing population trend is leading to land use intensification, with negative effects on several ecosystem services such as pollination (IPBES, 2016; United Nations, 2019). Human wellbeing is intimately linked to pollination, not only for the agri-food production and food security issues, but also for the quality of fruit resulting from this service (Classen et al., 2014; Smith et al., 2015; Stein et al., 2017; Elisante et al., 2020). In this context, the scientific community concurs that pollinators largely contribute to the sustainable development of the planet, being relevant in the achievement of United Nations Sustainable Development Goals (Patel et al., 2020). The efficiency of pollination is positively linked to the abundance and diversity of some insects, mainly bees and hoverflies (Dainese et al., 2019), which is in turn influenced by local and landscape variables. For example, bee richness was found to decline in agricultural sites that are surrounded by progressively higher built-up surfaces (Bennett and Lovell, 2019). A similar pattern was also observed considering agricultural intensification that causes a dramatic decrease of pollinator richness, following the loss of semi-natural patches surrounding the farms (Deguines et al., 2014).

To date, most of the pollinator-based research comes from Europe and North America, while significant data gaps occur for Asian and African regions that are currently experiencing an intense agricultural and industrial development (Timberlake and Morgan, 2018). Specifically, in Sub-Saharan Africa, the land use intensification through urban and agricultural expansion is increasing as fast as the population growth (Eckert et al., 2017; Sulemana et al., 2019). In Sub-Saharan countries, agriculture represents the main source of family sustainment (Stein et al., 2017) with about 80% of the population relying on subsistence farming in Tanzania (Sawe et al., 2020a). Moreover, with 80% of farms being smaller than 2 ha, these agriculture systems are mainly represented by smallholder farms (Garrity et al., 2010) that are widely diffused in urban and peri-urban landscapes (Armar-Klimesu, 2000). The spread of high commercial value pollination-dependent crops (e.g., coffee, watermelon, and beans; Gemmill-Herren et al., 2014) also makes these agricultural systems more susceptible to fluctuations in

terms of pollination service quality (Dainese et al., 2019).

Studies conducted in Ghana highlighted that urban farmland host lower abundances of pollinating insects compared to urban greenspaces (Guenat et al., 2019). Other studies addressed how pollination efficiency and different management conditions affect yield and quality of crops (Classen et al., 2014; Stein et al., 2017; Sawe et al., 2020b). Apart from these studies, the effect of land use intensification on pollinators was poorly investigated. However, a better and exhaustive understanding of plant-pollinator insect dynamics in Sub-Saharan smallholder farming systems is necessary to promote effective farm-scale solutions focusing on the management of biological features. For example, specific policy actions directed to the enhancement of available floral resources, such as the establishment of flower strips, are of primary concern. As already demonstrated in other studies, these strategies can increase the pollinators abundance (Jönsson et al., 2015), enhance crop-flower visitation (Feltham et al., 2015) and contribute to mitigate the impact of land use intensification.

One efficient way to describe ecosystem functioning is the implementation of network theory in the context of interactions between plants and pollinators (Biella et al., 2017). Recently, the identification of pollen taxonomy has been based on DNA metabarcoding approaches to characterize the composition of the pollen foraged by pollinator insects and to evaluate variation in plant-pollinator interactions (e.g., Biella et al., 2019; Macgregor et al., 2019). Although this approach could add valuable ecological details about the effects of land use change on biodiversity features (Adedoja and Kehinde, 2018), to the best of our knowledge, no similar studies were conducted employing this highly informative molecular-based method in Sub-Saharan Africa. Several case studies proved the suitability of this approach in other countries (Danner et al., 2017; Nürnberger et al., 2019) also because it requires relatively simple molecular-biology skills and allows to reduce the time spent for field observation while improving the number of observed interactions (Bell et al., 2017).

In this study, we combined a DNA metabarcoding-based approach with field monitoring and land use analysis to investigate how different features of surrounding landscape affect the plant-pollinator communities in smallholder farms of Northern Tanzania by quantifying multiple issues. Firstly, we characterized biodiversity parameters, and in particular, we evaluated how the species richness of bees and hoverflies, here selected for their importance as main

representers of the pollinators guild (Hennig and Ghazoul, 2012; Ssymank et al., 2008), was affected by land use intensification. Secondly, we characterized plant-pollinator interaction networks in response to landscape features. Overall, we aimed at verifying if local scale biodiversity features, that can be actively managed and promoted by landowners, could mitigate the negative effects of land use intensification. This is expected to decrease the habitat quality within the farm surroundings, due to the loss of nesting and foraging niches, and to the higher habitat fragmentation and isolation (Kovacs-Hostyanszki et al., 2017). For these reasons we hypothesized to find negative effects on plant and pollinators richness and alteration of the foraging preferences and structural properties of the interaction networks. Since these factors could imply relevant changes in the stability of the overall pollination service, with consequences at the agricultural production level, this assessment intended to provide a first baseline to drive and support reliable policies for a more sustainable development of the smallholder farming systems of Sub-Saharan Africa.

MATERIAL AND METHODS

Study sites and landscape description

The study was conducted in Northern Tanzania, in the area surrounding Mt. Meru and the southern-western slopes of Mt. Kilimanjaro. Specifically, the areas of the rural and urban district of the Arusha region and the rural areas of the Moshi and Hai districts of Kilimanjaro region were investigated. Within this study area, 27 smallholder farms (< 1 ha of occupied surface) were selected as sampling sites (Fig. 1 and Appendix A, Table S1). These were representative of different levels of land use intensification. In particular, the investigated farms ranged from those located in urban areas, with a prevalence of impervious surfaces in the surrounding, to those localized in highly exploited homogeneous agricultural landscapes that were mainly surrounded by large-scale cultivated lands. Finally, farms mainly surrounded by semi-natural landscapes, such as grassland and forest, were also selected as representative of the minimum disturbance conditions induced by land use intensification in Tanzania. The distance among study sites was at least of 1 km (a distance considered higher than the usual home range of most pollinator insects (Garibaldi et al., 2014)). To demarcate the boundaries of each farm we used a Garmin eTrex Venture HC GPS device (declared accuracy, 3 m). For each farm, we manually quantified the area of

different land uses by polygonizing satellite images in a 500 m radius buffer, corresponding to the expected mean foraging range of most bees and hoverflies (Fisogni et al., 2020). This assessment was conducted by using QGIS 3.4 with basemap Bing Aerial (OpenLayers QGIS plugin updated in 2018). The area of polygons that shared the same land use type was summed to calculate the total amount of impervious surfaces (e.g., houses, infrastructures, and cemented roads), cultivated land, and semi-natural land (i.e., natural, unmanaged patches, meadows and lawns). These land use categories were chosen to estimate urbanization (proportion of impervious land), as well as the degree of agricultural land use intensification (ratio between cultivated and semi-natural land). Furthermore, a Shannon-Wiener index of land use was calculated to understand the effects of land use heterogeneity on plant and pollinator communities. A visual check was performed in the field, prior to sampling activities, to verify the coherence between the results of land use categorization and the landscape features of the farms' surroundings (e.g., presence of infrastructures, agricultural fields or semi-natural areas). All the selected farms were characterized by field margins hosting wild or barely managed vegetation rich in flowering species. The natural remnant that surrounded the farms ranged from small patches of ornamental trees in the more urbanized areas, to large patches of forest or grassland in the farming sites showing low land use intensification. Fruit trees, such as avocado, and hedgerows were abundant in most of the farms, either for production or demarcation purposes. The main cultivated crops at the time of sampling were maize, beans, sorghum, but also strawberry and banana or leafy vegetables such as cabbage.

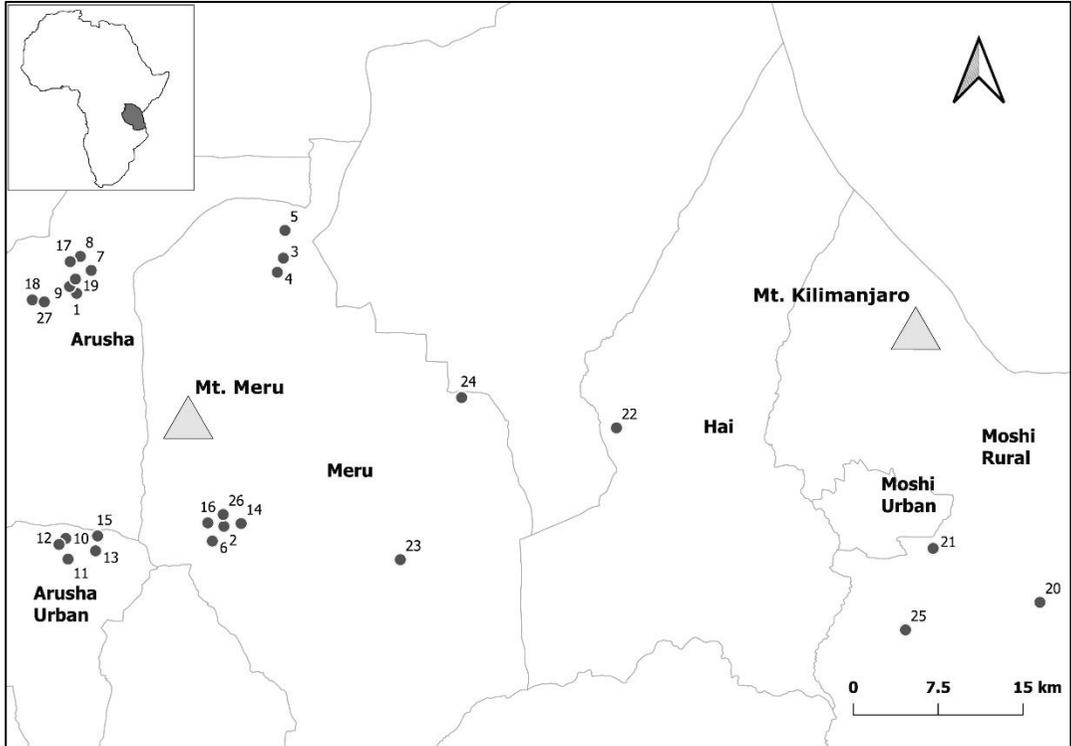


Fig. 1. Map of the study sites, further details about sampling methodology are reported in Appendix A, Table S1.

Characterization of pollinator and plant communities

Sampling activities were performed in June 2018 at the end of the wet season. This period is still favourable for agricultural production in Northern Tanzania and actually, many crops were still flowering during sampling, that was performed during sunny or moderately cloudy days. In the selected farms (see Appendix A, Table S1) insect and plant communities were investigated by using pan traps and net sampling (to assess the network interactions through DNA metabarcoding of pollen, see next sections) and vegetation quadrats, respectively. Six coloured pan traps (2 Blue, 2 Yellow and 2 White, 25 cm diameter and 10 cm depth), filled with 200 mL of water and 1 mL of soap, were placed with alternate colours at the margin of the cultivated area. The traps were placed directly on the ground, since the vegetation was low and most of the surrounding flowers were nearly at the same level. Furthermore, we carefully checked that all the traps were clearly visible from each side of the investigated area. The inter distance between each trap was approximately 10 m and the exposure time was 24 h. After collection, we

selected bees and hoverflies because of their well-known importance as flower visitors. These insects were assigned to morphospecies and used to estimate the abundance of flower visitors at each farm (i.e., the ratio between the number of flower visitor insects caught per site, and the total number of flower visitor insects at all sites).

Plant community species richness, plant cover (i.e., cm² of plant occupied surface), and floral abundance (i.e., the number of blooming flowers or inflorescences) were estimated at the field margins by using vegetation quadrats. Briefly, after a preliminary visual assessment of the field margins extension and heterogeneity of the flowering plant community, three to six vegetation quadrats (0.5*0.5 m) were performed. Furthermore, the ratio between flower abundance and plant cover was calculated as a measure of flower density.

Plant DNA reference database

A reference database of nuclear ITS2 sequences, comprehensive of the main flowering species occurring at the study sites, was produced as in Biella et al. (2019) to overcome DNA metabarcoding identification drawbacks posed by the unavailability of a local dataset of plant DNA barcodes. Briefly, some leaves of plant species observed in vegetational squares or occurring in the study sites were collected and subjected to DNA extraction and ITS2 amplification and sequencing. These specimens were stored in the herbarium of the Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy (herbarium code MIB: ZPL). A list of the plant taxa found into the vegetational quadrats, as well as information about reference sequences produced in this study are available in Appendix A, Table S2.

Pollen DNA metabarcoding

In order to obtain information about plant-pollinator interactions, the taxonomic composition of the pollen carried by insects was assessed through ITS2 DNA metabarcoding. To do this, only insects collected through an entomological net and observed foraging on flowers were analysed. At each farm, the insect capture sessions were performed for 1 h of sampling in a ~ 50 50 m subplot (Appendix A, Table S1). Sampling was performed between 7:30 am and 5:30 pm and to prevent cross contamination a clean net was used at each farm. Each individual was stored in single plastic tubes filled with 70% ethanol.

To remove pollen grains from the collected pollinators, tubes containing ethanol 70% and the insects were vigorously vortexed for 10 s. Afterwards, insects were removed from the tubes and the preserving ethanol was centrifuged at 14,000 rpm for 10 min to allow the pollen grains deposition. Ethanol was completely removed through evaporation under a chemical hood.

Pollen DNA extraction and sequencing details are reported in appendix A, Supplementary Text S1. Briefly, the samples were grinded, and DNA was extracted according to standard protocols. The internal transcribed spacer 2 (ITS2) region was amplified using primers S2F and S3R (Chen et al., 2010) with the addition of the Illumina overhang sequence adapters. Library preparation and sequencing were performed through the Illumina MiSeq instrument using MiSeq 600 V3 (2 300-bp paired-end sequencing). The obtained reads were paired, pre-processed, and clustered in Exact Sequence Variants ESVs (Callahan et al., 2017) following a standard bioinformatic pipeline prior to taxonomic assignment (See Appendix A, Supplementary Text S1 for taxonomic assignment and further details). After this treatment, the insects were identified at the best taxonomic level and this information was also coupled with that coming from the pantrapped insects to evaluate the overall pollinator species richness at the sampling sites (further details about the identification of net collected insects involved in plant-pollinator network analysis are available in Appendix A, Table S4).

Interaction networks

Matrices of interactions between pollinator individuals (single pollinator insects) and plant species were built to calculate network indices at the site (farm) level. This approach allows to describe changes in the foraging choices of pollinators (Tur et al., 2015; Biella et al., 2019) and is informative of the possible effects of land use change and other anthropogenic stressors on biodiversity (Adedoja and Kehinde, 2018). Indices of network-level complementary specialization, foraging overlap in the pollen resources carried by insects (i.e., pollinator foraging niche overlap), and network size were calculated through the R package bipartite (Dormann et al., 2008). Network complementary specialization is a network-level index that ranges from 0 (no specialisation) to 1 (complete specialisation). Low complementary specialization is usually related to low functional redundancy in flower visitation (Kaiser-Bunbury and Blüthgen, 2015). To allow a more intuitive comparison of H2' with the pollinator foraging niche overlap, we calculated 1-H2'

as a measure of complementary generalization (so that zero corresponds to complete specialization and one to no specialization). This index has been shown to be robust against sampling intensity and network size, making it a useful tool for the comparison of networks across multiple habitats (Classen et al., 2020). To calculate this index, the number of DNA reads was used as a measure of the abundance of the pollen carried by an individual and then as the weight of plant-pollinator interactions as in Biella et al., 2019. The pollinator foraging niche overlap was inferred as the mean similarity in interaction patterns among individuals. This index was calculated as the Jaccard similarity index among pollinators in terms of plants found on their body. Finally, the pollinator group level mean was then derived. Values tending 0 s indicate low or no common use of plants, while 1 s indicates perfect overlap and thus foraging choices converging on few resources. Network size was calculated as the product between the number of animal species and the number of plant species in the matrix of interactions and used as a predictor to account for the role of network size variation on the network indices as in Olesen and Jordano (2002) and as in Biella et al. (2020).

Statistical analysis

To evaluate the effects of local biotic features (i.e., flower density and abundance, plant cover and flower visitors abundance) and of land use intensification covariates, such as urbanization (i.e., proportion of impervious land), agricultural land use intensification (i.e. ratio of cultivated-to-natural land) and land use heterogeneity on the investigated response variables (i.e., pollinator and plant species richness, pollen sample species richness and proportion of crop pollen and network indices), we used a Generalized Linear Models (GLM) regression approach. To exclude correlation among the covariates included in the models, the vif function in the car R package (i.e., variance inflation factor with an exclusion threshold of 3) was used. Furthermore, a visual validation approach was employed to evaluate the use of logarithm or square root transformations on covariates to improve the goodness of fit between covariates and response variables (details about the used transformation are reported in Table 1). The few missing data were either removed or replaced with simple random imputation (Kadengye et al., 2012). The regression models were largely built on ecological expectation bases. In detail, we expected that the considered response variables

would have been influenced by the covariates of land use intensification that were included in all the evaluated models. Different local biotic features were included as covariates in the models. Specifically, flower abundance and flower visitor abundance were included as covariates in the models related to i) pollen species richness, ii) proportion of crop pollen collected, and iii) network indices. This is because it is reasonable to expect that the availability of floral resources and the abundance of insects that compete for them are important drivers of competition between individuals. This competition should lead differences in insects' foraging preferences (pollen composition features) and strategies (network indices) (Araújo et al., 2011; Fontaine et al., 2008). The flower density was used as a covariate to predict variation in pollinator species richness. As a matter of fact, high resource density conditions are known to be highly attractive for pollinators, thus they were also expected to improve species richness (Hegland and Boeke, 2006; Vrdoljak et al., 2016). Concerning plant richness, the effect of plant cover was investigated since it could be expected that species richness increases where the cover is higher (Sanaei et al., 2018). Details on the distribution applied in regression models are reported in Table 1.

The log likelihood ratio test was used to test predictor significance ($P < 0.05$), while the Moran test was applied to confirm the absence of spatial autocorrelation within data. This test revealed only non-significant results ($P > 0.05$), thus indicating that no serious spatial autocorrelation occurred in the dataset. All the analyses were performed using R (version 3.6.1; R CoreTeam 2019).

RESULTS

Characterization of pollinator and plant communities

Overall, 141 flower visitors belonging to 27 morphospecies, with 91.5% of the individuals classified as bees, were collected through pan traps. Moreover, 264 flower visitors belonging to 56 taxa with 87.5% of the individuals classified as bees, were collected through net sampling (Appendix A, Table S4). Seventy-three plant taxa were identified from the vegetation survey (Appendix A, Table S2). Asteraceae (27.39%) and Fabaceae (9.5%) were the most represented families. About 22% of the identified plants belonged to non-native taxa, with exotic species such as *Ageratum conyzoides*, *Argemone mexicana*, *Lantana camara*, *Datura stramonium* and *Parthenium hysterophorus* largely represented in almost

all the visited farms.

Pollinator species richness was negatively related to the amount of impervious land (Fig. 2a) and to the ratio between cultivated and natural land (Fig. 2b) but positively related to flower density (Fig. 2c). Plant richness was positively related to land use heterogeneity (Appendix A, Fig. S1-a) and plant cover (Appendix A, Fig. S1-b). Regression model details are reported in Table 1 - section Pollinator and plant communities.

Pollen DNA metabarcoding and interaction networks

The HTS sequencing yielded 18,506,952 reads (mean 41,772.87 reads per sample), after raw sequence processing 1778 ESVs were obtained, and assigned to 149 plant taxa, with 70.5% of the assignment at species level. The mean number of pollen taxa per insect was 4.2 ± 2.9 (range 1–18 plant taxa).

As reported in Table 1 (section Pollen composition analysis), the species richness in pollen samples was not significantly related to landscape or local covariates. Conversely, the collection of pollen from crop species was higher in landscapes with a high proportion of cultivated land (Appendix A, Fig. S1-c).

Concerning the network metrics (Table 1), the pollinator foraging niche overlap significantly increased in the farms with higher proportions of impervious surface (Fig. 3a) and agricultural land use intensification expressed as cultivated-to-natural land ratio (Fig. 3b), while it was negatively affected by flower abundance (Fig. 3c). The Complementary generalization ($1-H2'$) significantly increased with the abundance of flower visitors (Fig. 3d) and decreased with flower abundance. Finally, the network size negatively affected pollinator foraging niche overlap and positively impacted network generalization. Additional details about the calculated network indices for each investigated farm are available in Appendix A, Table S3.

Table 1: Results of the final regression models analysis of pollinator species richness, plant species richness, pollen species richness, proportion of crop pollen, pollinator foraging niche overlap and generalization (1-H2') as function of both landscape and local covariates. β_i : regression coefficient; χ^2 : log-likelihood ratio test; p : p-values (significant value in bold).

Section	Variables	Covariates	β_i	χ^2	p
Pollinator -plant communities	Pollinator species richness	log (prop impervious land+1)	-6.96	9.43	0.002
		log (flower density*100)	0.34	5.40	0.02
	Plant species richness	sqrt (cultivated/natural land)	-0.71	4.55	0.03
		log (land use heterogeneity+1)	1.97	0.39	0.19
		log (prop impervious land+1)	-0.01	0.01	0.92
		sqrt (cultivated/natural land)	-0.07	0.24	0.61
		log (land use heterogeneity+1)	1.14	3.47	0.06
		log (plant cover+1)	0.30	5.28	0.02
Pollen composition analysis	Pollen species richness	log (prop impervious land+1)	0.03	0.19	0.66
		log (cultivated/natural land+1)	-0.02	0.03	0.86
		log (flower abundance +1)	-0.05	0.09	0.76
		log (flower visitors abundance+1)	-0.48	0.13	0.71
	Proportion of crop pollen	log (prop impervious land+1)	0.12	0.45	0.50
		log (cultivated/natural land+1)	0.59	4.01	0.05
		log (flower abundance +1)	0.02	0.00	0.97
		log (flower visitors abundance+1)	-5.66	0.82	0.36
Interaction network metrics	Pollinator foraging niche overlap	log (prop impervious land+1)	2.52	14.6	< 0.001
		log (cultivated/natural land+1)	1.48	6.64	0.001
		sqrt (flower abundance)	-2.27	4.27	< 0.001
		log (flower visitors abundance+1)	2.12	0.02	0.86
		network size	-5.85	45.3	< 0.001

Section	Variables	Covariates	β_i	χ^2	p
1-H2'		log (prop impervious land+1)	2.31	0.80	0.37
		log (cultivated/natural land+1)	-0.06	0.08	0.76
		sqrt (flower abundance)	0.08	5.66	0.01
		log (flower visitors abundance+1)	8.29	3.6	0.05
		network size	0.001	3.9	0.04

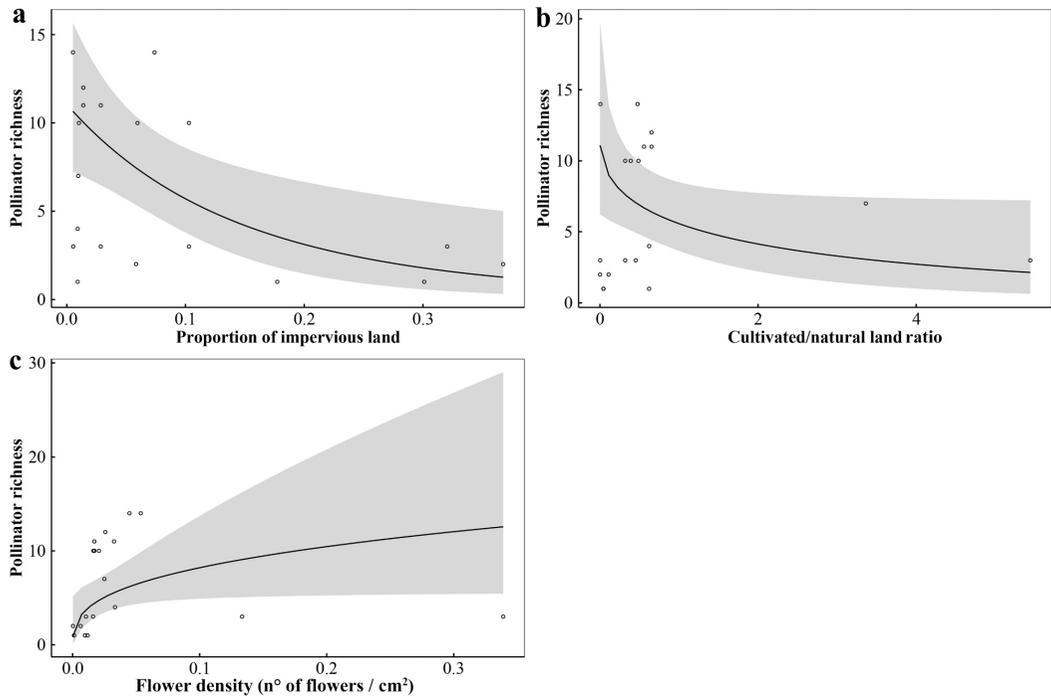


Fig. 2. Regression analysis of pollinator richness as a function of the proportion of impervious land (a), ratio between cultivated and natural land (b) and flower density (n° of flowers/cm²) (c).

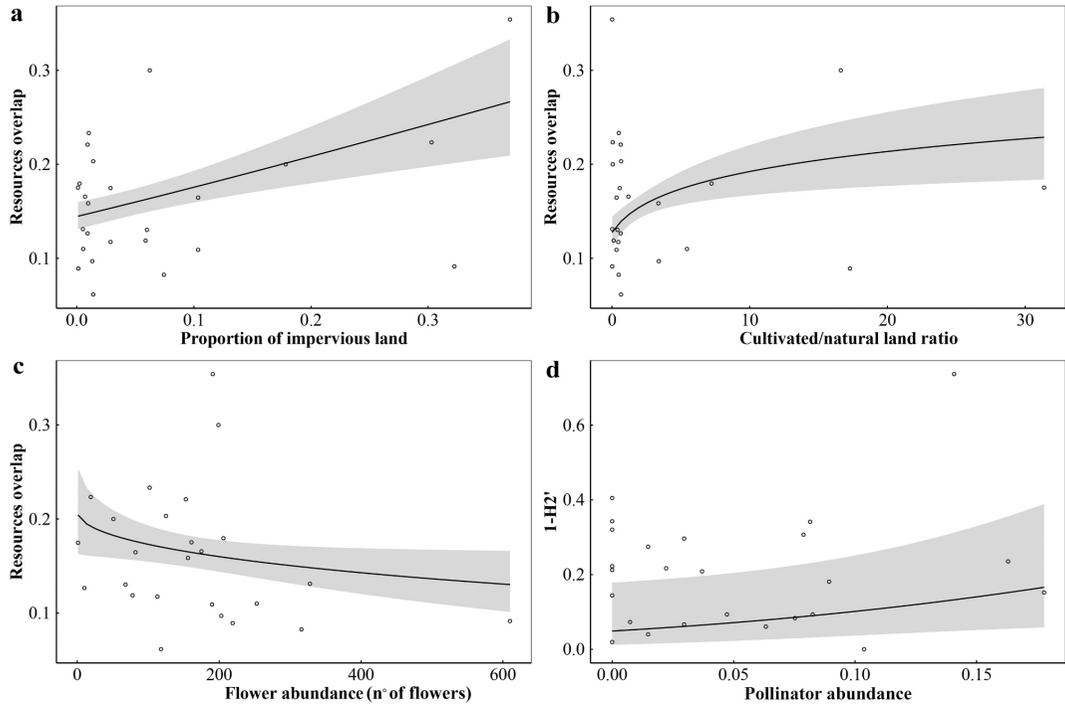


Fig. 3. Regression analysis of pollinator foraging niche overlap (resource overlap) as a function of the proportion of impervious land (a), cultivated/natural land ratio (b) and flower abundance (n° of flowers) (c). The Plot (d) represents the regression output between complementary generalization -H2' and flower visitor abundance.

DISUSSION

In this study, we shed light on the ways by which plant and pollinator insect assemblages, and the interactions between them, are influenced by land use intensification and by local features of resource availability and abundance of flower visitor insects at smallholder farms in Northern Tanzania. This region represents a clear example of the intense spread of anthropogenic landscapes that urban and rural areas of several Sub-Saharan African regions are facing. Apart from conventional field investigations, we exploited the high-resolution power of DNA meta-barcoding to successfully characterize the foraging preferences of pollinators. In this context, the building of a dedicated DNA reference database, representative of the local plant biodiversity, facilitated the success rate and the reliability of taxonomic identification of pollen samples. This has also been confirmed by other studies showing that an integrated molecular-field approach is useful for expanding the amount of information from field sampling activities (Biella et al., 2019; Elliott et al., 2020). Based on our results, we also recommended the use of this approach for future studies requiring the analysis of pollen samples, especially in countries where the local biodiversity has already not been fully characterized.

In this survey, the standard field monitoring approaches highlighted that at the investigated farms, the pollinator richness was negatively affected by the amount of impervious surfaces in the surrounding landscape. This result confirms a previous research from West African farming systems (Guenat et al., 2019). Reasons for this trend could be found in the increase of impervious surface, responsible for the contraction of green spaces and for the increase of their isolation. This, in turn, leads to a landscape characterized by discontinuous and intermittent distribution of floral resources, a condition that could result into locally poor pollinator assemblages, due to resources that are difficult to access (Egerer et al., 2020). Similarly, agricultural land use intensification is expected to reduce habitat quality and leads to a simplified landscape with lower habitat diversity and availability (Deguines et al., 2014), and this could explain the reduction in pollinator richness observed in response to the increase of agricultural surfaces.

From the pollen analysis, we found that the proportion of pollen of crops increased with cultivated-to-natural land ratio. This phenomenon could be justified by considering the possible “pollinator magnet” effect of mass flowering

crops occurring in rural landscapes, where the cultivated species largely overwhelm the abundance of flower resources offered by the spontaneous ones (Gilpin et al., 2019). Hence, in the context of smallholder farms, flowering crops might play an important role as food resources for pollinator insects. In heavily cultivated areas, the generalist pollinator species might be more abundant and advantaged instead of the specialist ones because of the higher propensity to change their foraging preferences for exploiting the available crop resources. Thus, changes in the pollinator community composition could also explain the higher collection of crop pollen in response to increased cultivated-to-natural land ratio.

A neutral relationship with agricultural land use intensification was found for plant richness. This observation disagrees with other studies indicating detrimental effects of the agricultural landscape on the composition and complexity of floral communities (Nicholls and Altieri, 2013). Conversely to what conventionally practiced in intensive agriculture, many smallholder farmers in Tanzania still manually extirpate weeds (personal observation), thus avoiding agrochemical runoff. This manual practice contributes to maintain the floral resources offered by wild plants at the field margins. Interestingly, plant species richness was higher at farms characterized by a higher plant coverage. Our result further contrasts with the expected simplification of the flowering plant community in agricultural landscapes (Hall et al., 2020) that should promote the abundance of the more competitive species, even in conditions of high plant coverage. This supports the possibility that smallholder agroecosystems could host an unexpectedly high flowering plant biodiversity with consequent benefit for pollinators (Fründ et al., 2010; Ouvrard et al., 2018). The relevance of biodiversity friendly local-scale practices on pollinator diversity was also found in other studies centred in Africa (Delaney et al., 2020). Hence, policies and management promoting farm-level plant cover will scale up to sustaining highly diverse pollinator communities, fostering the small-scale ecological intensification of smallholder farms.

Local diversity influences biotic interactions among organisms, and alterations of the environment will also alter network structures by filtering species assemblages and driving which interactions occur (Biella et al., 2020). The adoption of DNA metabarcoding to characterize such interactions, allowed us to evaluate fine changes in the foraging preference of pollinators, but also to highlight changes in the structure of plant-pollinator networks in response to land

use and local resource availability. Considering individuals instead of species-level interactions allows us to consider intraspecific behavioral variation and to account for early impacts of changing foraging contexts and alterations of competitive dynamics that might otherwise be overlooked (Ings et al., 2009; Araújo et al., 2010). Positive relationships between the overlap in transported pollen resources (an indication of foraging niche overlap) and the landscape features were observed in this study for the urban and agricultural surfaces. In both cases this trend could be the results of an increased community of generalist pollinators or could reflect low diversity in plant communities of urban areas and intensified agricultural landscapes, which led pollinator individuals to converge on the available floral resources. In particular, more intensified rural sites do not only mean mass flowering crops, but also implies lower cover of the natural land use and hence less non-crop floral resources at the landscape-level. This condition is also supported by the higher amount of crop species found in pollen samples also by a previous study (e.g., Pornon et al., 2019). The mechanism by which the amount of resources determines foraging niche convergence is evident not only at the landscape but also at the local scale, where higher floral abundance led to a higher foraging niche complementarity (Blüthgen and Klein, 2011) and hence to a reduction in the overlap of the resources foraged by insects. This result is confirmed by the reduction of complementary generalization observed in response to flower abundance and fits well within the framework of the Optimal Foraging Theory, for which foragers are expected to converge on the available resources when plant abundance is low (Fontaine et al., 2008; Biella et al., 2019). Furthermore, the complementary generalization index was found to be significantly affected by the abundance of flower visitors, with higher generalism at the network level in conditions of high abundance of flower visitors. A possible explanation of this phenomenon is that more competitive conditions may lead to a faster depletion of floral resources. According to the optimal foraging theory (OFT), this condition could drive to a diversification and/or expansion of the pollinators' diet, possibly increasing (Araújo et al., 2011) the generalism of the foraged plants. Although specific investigations are necessary to address this issue, the observed increase of network level generalization, could reflect a higher functional redundancy and stability of interactions (Kaiser-Bunbury and Blüthgen, 2015). These considerations further support the necessity of enhancing pollinator

insects abundance in Sub-Saharan farming contexts (e.g., by means of ecological intensification; Kovács-Hostyánszki et al., 2017).

CONCLUSIONS

Our study pointed out that increasing urbanization and agricultural cover reduces plant and pollinator biodiversity and negatively impacts the complexity of their interactions. Conversely, the local-scale availability of floral resources has shown positive effects in buffering pollinator decline and mitigating all the detrimental effects induced by land use intensification phenomena in the Sub-Saharan context. Thus, our study clearly highlights the importance of policies and managements targeting small-scale measures aiding local biodiversity. Based on our first evidence, such policies should include actions aimed at improving the presence of green spaces in urban landscape to break the continuity of impervious coverage and maintaining high habitat heterogeneity and seminatural spaces in rural landscapes. Good practices in the management of both private and public greenspaces and agroforestry (e.g., reducing mowing frequency, planting flower strips, and encouraging rotation strategies by introducing pollinator forage crops), should be fostered by administrations to reduce the risks related to the loss of pollinators and thus of the pollination service. Actions to preserve pollinators is therefore pivotal in Sub-Saharan farming systems to achieve some of the United Nations SDGs and to reduce human nutritional deficits (Smith et al., 2015; Patel et al., 2020) in a framework of ‘one health’ concept, for which the health of people is closely connected to the health of biodiversity and ecosystems where they live.

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CRedit authorship contribution statement

Nicola Tommasi: Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. Paolo Biella: Methodology, Investigation, Formal analysis, Writing - review & editing. Lorenzo Guzzetti: Methodology, Investigation,

Formal analysis. Julius Vincent Lasway: Investigation, Writing - review & editing. Henry Kenneth Njovu: Investigation, Writing - review & editing. Andrea Tapparo: Investigation. Giulia Agostinetto: Software, Visualization. Marcell Karl Peters: Writing - review & editing. Ingolf Steffan-Dew- enter: Writing - review & editing. Massimo Labra: Funding acquisition, Conceptualization, Writing - review & editing. Andrea Galimberti: Funding acquisition, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

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

Data Availability

The dataset generated through DNA metabarcoding in this study was submitted to the EBI metagenomics portal (<https://www.ebi.ac.uk/metagenomics/>). BioSamples accessions are: PRJEB41466 (ERP125246). ITS2 DNA barcode sequences are available on GenBank with the accession number MZ489668 - MZ489808. All relevant data are within the paper or stored in a public repository (<http://doi/10.6084/m9.figshare.13637576>).

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Appendix A. Supplementary data

Table S1: Sampling details (district and geographic coordinates) of all the investigated farms and sampling methodology (pan trap, vegetation quadrats and net sampling).

Farm ID	Administrative Region	Latitude	Longitude	Net sampling	Pantrap-vegetation quadrat
1	Arusha rural district	-3.1767	36.7024	yes	yes
2	Meru district	-3.3616	36.8145	yes	yes
3	Meru district	-3.1486	36.8669	yes	-
4	Meru district	-3.1599	36.8621	yes	yes
5	Meru district	-3.1265	36.8682	yes	yes
6	Meru district	-3.3742	36.8104	yes	yes
7	Arusha rural district	-3.1584	36.7140	yes	yes
8	Arusha rural district	-3.1472	36.7054	yes	yes
9	Arusha rural district	-3.1712	36.6968	yes	yes
10	Arusha urban district	-3.3721	36.6938	yes	yes
11	Arusha urban district	-3.38858	36.69557	yes	yes
12	Arusha urban district	-3.3769	36.6884	yes	yes
13	Arusha urban district	-3.3820	36.7175	yes	yes
14	Meru district	-3.3602	36.8333	yes	yes
15	Arusha urban district	-3.3701	36.7191	yes	yes
16	Meru district	-3.3597	36.8068	yes	yes
17	Arusha rural district	-3.1514	36.6973	yes	yes
18	Arusha rural district	-3.1819	36.6671	yes	yes
19	Arusha rural district	-3.1653	36.7015	yes	yes
20	Moshi rural district	-3.4230	37.4690	yes	-
21	Moshi rural district	-3.3800	37.3840	yes	-
22	Kilimanjaro Hai district	-3.2840	37.1320	yes	-
23	Meru district	-3.3890	36.9600	yes	-

Farm ID	Administrative Region	Latitude	Longitude	Net sampling	Pantrap-vegetation quadrat
24	Meru district	-3.2500	37.0090	yes	-
25	Moshi rural district	-3.4450	37.3620	yes	-
26	Meru district	-3.3570	36.8163	-	yes
27	Arusha rural district	-3.1835	36.6767	-	yes

Table S2: List of the plant taxa identified during vegetation sampling. Plants used to produce the ITS2 DNA reference database are also indicated, as well as NCBI submitted voucher name. In the last column T indicates tree/woody species while H indicates herbs.

Family	Genus	Species	Vegetation quadrat	ITS2 Sequence produced in this study	Voucher name	Tree/Herbs
Asteraceae	<i>Bidens</i>	<i>Bidens pilosa</i>	x	x	MIB:SASS_0001	H
Commelinaceae	<i>Commelina</i> <i>sp1</i>	-	x	x	MIB:SASS_0002	H
Fabaceae	<i>Acacia</i> sp	-	x	-		T
Asteraceae	<i>Ageratum</i>	<i>Ageratum conyzoides</i>	x	x	MIB:SASS_0003	H
Amaranthaceae	<i>Amaranthus</i> sp	-	x	x	MIB:SASS_0004	H
Asteraceae	<i>Galinsoga</i>	<i>Galinsoga parviflora</i>	-	x	MIB:SASS_0005	H
Asteraceae	<i>Ageratum</i> sp	-	x	x	MIB:SASS_0006	H
Asteraceae sp4	-	-	x	x	MIB:SASS_0009	H
Acanthaceae	<i>Thunbergia</i>	<i>Thunbergia alata</i>	-	x	MIB:SASS_0010	H
Commelinaceae	<i>Commelina</i>	<i>Commelina communis</i>	-	x	MIB:SASS_0011	H
Fabaceae	<i>Aeschynomene</i>	<i>Aeschynomene schimperi</i>	x	x	MIB:SASS_0013	H
Convolvulaceae	<i>Ipomoea</i>	<i>Ipomoea obscura</i>	-	x	MIB:SASS_0014	H
Asteraceae	<i>Sonchus</i> sp	-	x	x	MIB:SASS_0017	H
Oxalidaceae	<i>Oxalis</i> sp	-	x	x	MIB:SASS_0020	H
Commelinaceae	<i>Commelina</i> sp	-	x	-		H
Malvaceae sp1	-	-	-	x	MIB:SASS_0022	H
Asteraceae	<i>Tagetes</i>	<i>Tagetes minuta</i>	x	x	MIB:SASS_0027	H
Verbenaceae	<i>Lantana</i>	<i>Lantana camara</i>	x	x	MIB:SASS_0028	H
Asteraceae	<i>Senecio</i>	<i>Senecio hadiensis</i>	-	x	MIB:SASS_0031	H

Family	Genus	Species	Vegetation quadrat	ITS2 Sequence produced in this study	Voucher name	Tree/Herbs
Acanthaceae sp1	-	-	x	x	MIB:SASS_0032	H
Solanaceae	<i>Datura</i>	<i>Datura stramonium</i> (A)	x	x	MIB:SASS_0036	H
Asteraceae	<i>Galinsoga</i> sp	-	-	-		H
Fabaceae	<i>Sesbania</i> sp	-	-	x	MIB:SASS_0037	T
Asteraceae sp5	-	-	-	x	MIB:SASS_0038	H
Acanthaceae	<i>Asystasia</i> sp	-	x	x	MIB:SASS_0040	H
Acanthaceae	<i>Justicia</i> sp	-	x	x	MIB:SASS_0042	H
Solanaceae	<i>Solanum</i>	<i>Solanum lycopersicum</i>	x	x	MIB:SASS_0046	H
Asteraceae sp6	-	-	x	x	MIB:SASS_0049	H
Asteraceae	<i>Hirpicium</i>	<i>Hirpicium diffusum</i>	x	x	MIB:SASS_0051	H
Lamiaceae	<i>Leucas</i> sp	-	x	x	MIB:SASS_0052	H
Malvaceae	<i>Abutilon</i> sp 1	-	-	x	MIB:SASS_0053	H
Malvaceae	<i>Sida</i> sp	-	-	x	MIB:SASS_0055	H
Fabaceae	<i>Crotalaria</i> sp	-	x	x	MIB:SASS_0056	H
Cucurbitaceae	<i>Cucumis</i>	<i>Cucumis dipsaceus</i>	-	x	MIB:SASS_0058	H
Brassicaceae sp1	-	-	x	-		H
Malvaceae	<i>Abutilon</i> sp 2	-	-	x	MIB:SASS_0059	H
Lamiaceae sp2	-	-	-	-		H
Asteraceae	<i>Spilanthes</i>	<i>Spilanthes paniculata</i>	-	x	MIB:SASS_0063	H
Malvaceae sp2	-	-	-	x	MIB:SASS_0065	H
Malvaceae	<i>Malvastrum</i>	<i>Malvastrum coromandelianum</i> (A)	-	x	MIB:SASS_0066	H
Fabaceae	<i>Calliandria</i> sp	-	-	x	MIB:SASS_0069	T
Fabaceae	<i>Phaseolus</i>	<i>Phaseolus vulgaris</i>	-	x	MIB:SASS_0070	H
Amaranthaceae	<i>Celosia</i>	<i>Celosia argentea</i>	-	-		H
Fabaceae	<i>Desmodium</i>	<i>Desmodium tortuosum</i>	-	x	MIB:SASS_0071	H
Lamiaceae	<i>Salvia</i> sp 3	-	-	x	MIB:SASS_0072	H
Rubiaceae	<i>Spermacoce</i>	<i>Spermacoce remota</i>	x	x	MIB:SASS_0073	H
Solanaceae	<i>Solanum</i>	<i>Solanum nigrum</i>	-	x	MIB:SASS_0074	H
Cannaceae	<i>Canna</i> sp	-	-	x	MIB:SASS_0075	H

Family	Genus	Species	Vegetation quadrat	ITS2 Sequence produced in this study	Voucher name	Tree/Herbs
Solanaceae	-	-	-	x	MIB:SASS_0077	H
Asteraceae	<i>Tagetes</i>	<i>Tagetes erecta</i>	-	x	MIB:SASS_0078	H
Lamiaceae	<i>Salvia sp 2</i>	-	-	x	MIB:SASS_0079	H
Lamiaceae	<i>Salvia sp 1</i>	-	-	x	MIB:SASS_0081	H
Acanthaceae sp2	-	-	-	x	MIB:SASS_0084	H
Convolvulaceae sp1	-	-	x	-		H
Cucurbitaceae	<i>Cucurbita sp</i>	-	-	x	MIB:SASS_0086	H
Asteraceae	<i>Emilia sp</i>	-	x	x	MIB:SASS_0088	H
Solanaceae	<i>Physalis sp</i>	-	-	x	MIB:SASS_0090	H
Verbenaceae	<i>Lantana sp</i>	-	-	-		H
Solanaceae	<i>Capsicum</i>	<i>Capsicum annum</i>	-	x	MIB:SASS_0093	H
Boraginaceae	<i>Heliotropium</i>	<i>Heliotropium steudneri</i>	x	x	MIB:SASS_0094	H
Lamiaceae sp3	-	-	-	x	MIB:SASS_0096	H
Lamiaceae sp4	-	-	-	x	MIB:SASS_0098	H
Asteraceae sp7	-	-	x	x	MIB:SASS_0099	H
Malvaceae sp3	-	-	-	x	MIB:SASS_0100	H
Fabaceae	<i>Senna sp 1</i>	-	-	x	MIB:SASS_0101	T
Fabaceae	<i>Senna sp 2</i>	-	-	x	MIB:SASS_0103	T
Solanaceae	<i>Datura</i>	<i>Datura stramonium</i> (B)	-	x	MIB:SASS_0104	H
Acanthaceae sp3	-	-	-	x	MIB:SASS_0105	H
Papaveraceae	<i>Argemone</i>	<i>Argemone mexicana</i>	x	x	MIB:SASS_0108	H
Asteraceae	<i>Schkuhria</i>	<i>Schkuhria pinnata</i>	-	x	MIB:SASS_0109	H
Solanaceae	<i>Nicandra sp</i>	-	-	x	MIB:SASS_0111	H
Malvaceae	<i>Hibiscus sp 2</i>	-	-	x	MIB:SASS_0116	H
Fabaceae	<i>Crotalaria sp 2</i>	-	x	x	MIB:SASS_0117	H
Commelinaceae	<i>Commelina</i>	<i>Commelina benghalensis</i>	-	x	MIB:SASS_0118	H
Fabaceae	<i>Lablab</i>	<i>Lablab purpureus</i>	-	x	MIB:SASS_0119	H
Solanaceae	<i>Solanum</i>	<i>Solanum tuberosum</i>	-	x	MIB:SASS_0121	H
Euphorbiaceae	<i>Euphorbia</i>	<i>Euphorbia pulcherrima</i>	-	x	MIB:SASS_0122	H

Family	Genus	Species	Vegetation quadrat	ITS2 Sequence produced in this study	Voucher name	Tree/Herbs
Fabaceae	<i>Lupinus sp</i>	-	-	x	MIB:SASS_0126	H
Lamiaceae sp5	-	-	-	-		H
Linaceae sp	-	-	-	x	MIB:SASS_0129	H
Asteraceae	<i>Kleinia sp</i>	-	-	x	MIB:SASS_0130	H
Asteraceae	<i>Gutenbergia sp</i>	-	x	-		H
Campanulaceae	<i>Campanula sp</i>	-	-	x	MIB:SASS_0131	H
Fabaceae	<i>Crotalaria sp 4</i>	-	-	x	MIB:SASS_0135	H
Lamiaceae sp6	-	-	-	-		H
Malvaceae	<i>Hibiscus sp 3</i>	-	-	-		H
Malvaceae	<i>Hibiscus sp</i>	-	x	-		H
Lamiaceae sp7	-	-	-	-		H
Solanaceae	<i>Solanum sp 1</i>	-	-	x	MIB:SASS_0137	H
Nyctaginaceae	<i>Mirabilis sp</i>	-	-	x	MIB:SASS_0139	H
Brassicaceae	<i>Capsella</i>	<i>Capsella bursa-pastoris</i>	-	x	MIB:SASS_0142	H
Asteraceae	<i>Tithonia</i>	<i>Tithonia diversifolia</i>	x	x	MIB:SASS_0367	H
Acanthaceae sp4	-	-	-	x	MIB:SASS_0369	H
Asteraceae sp8	-	-	-	x	MIB:SASS_0370	H
Rubiaceae	<i>Spermacoce sp</i>	-	-	x	MIB:SASS_0372	H
Lamiaceae sp8	-	-	-	-		H
Brassicaceae sp2	-	-	-	x	MIB:SASS_0374	H
Fabaceae	<i>Vigna sp</i>	-	-	x	MIB:SASS_0378	H
Asteraceae	<i>Bidens sp</i>	-	x	-		H
Malvaceae	<i>Malvastrum</i>	<i>Malvastrum coromandelianum</i> (B)	x	x	MIB:SASS_0379	H
Apocynaceae	<i>Asclepias sp</i>	-	-	x	MIB:SASS_0382	H
Orobanchaceae	<i>Sopubia</i>	<i>Sopubia lanata</i>	-	x	MIB:SASS_0384	H
Caprifoliaceae	<i>Sambucus sp</i>	-	-	x	MIB:SASS_0386	H
Fabaceae	<i>Pseudarthritis</i>	<i>Pseudarthritis panii</i>	-	x	MIB:SASS_0387	H
Solanaceae	<i>Solanum sp 2</i>	-	-	x	MIB:SASS_0390	H
Scrophulariaceae	-	-	-	-		H

Family	Genus	Species	Vegetation quadrat	ITS2 Sequence produced in this study	Voucher name	Tree/Herbs
sp						
Verbenaceae	<i>Verbena</i>	<i>Verbena officinalis</i>	-	x	MIB:SASS_0392	H
Verbenaceae	<i>Duranta</i>	<i>Duranta erecta</i>	-	x	MIB:SASS_0393	H
Lamiaceae	<i>Leonotis sp</i>	-	x	-		H
Asteraceae sp9	-	-	x	x	MIB:SASS_0395	H
Asteraceae	<i>Calotis</i>	<i>Calotis scabiosifolia</i>	-	x	MIB:SASS_0397	H
Asteraceae sp1	-	-	x	x	MIB:SASS_0398	H
Poaceae	<i>Lolium sp</i>	-	x	-		H
Fabaceae	<i>Indigofera</i>	<i>Indigofera arrecta</i>	x	x	MIB:SASS_0399	H
Boraginaceae sp	-	-	x	x	MIB:SASS_0401	H
Convolvulaceae	<i>Ipomoea sp</i>	-	-	x	MIB:SASS_0402	H
Asteraceae	<i>Aster sp</i>	-	-	x	MIB:SASS_0405	H
Fabaceae	<i>Indigofera sp</i>	-	-	x	MIB:SASS_0406	H
Campanulaceae sp	-	-	-	x	MIB:SASS_0408	H
Caprifoliaceae	<i>Lonicera sp</i>	-	-	-		H
Acanthaceae	<i>Hypoestes sp</i>	-	x	x	MIB:SASS_0410	H
Nyctaginaceae	<i>Mirabilis</i>	<i>Mirabilis jalapa</i>	x	-		H
Oleaceae	<i>Jasminum</i>	<i>Jasminum elongatum</i>	-	x	MIB:SASS_0411	H
Cyperaceae	<i>Cyperus</i>	<i>Cyperus exilis</i>	x	x	MIB:SASS_0413	H
Malvaceae sp4	-	-	-	x	MIB:SASS_0414	H
Asteraceae sp10	-	-	-	x	MIB:SASS_0419	H
Asteraceae sp11	-	-	-	x	MIB:SASS_0420	H
Asteraceae	<i>Parthenium</i>	<i>Parthenium hysterophorus</i>	x	-		H
Euphorbiaceae	<i>Euphorbia sp</i>	-	x	x	MIB:SASS_0421	H
Orchidaceae	<i>Eulophia</i>	<i>Eulophia streptopetala</i>	-	x	MIB:SASS_0422	H
Fabaceae sp	-	-	-	x	MIB:SASS_0423	H
Euphorbiaceae	<i>Croton</i>	<i>Croton megalocarpus</i>	-	x	MIB:SASS_0425	T
Portulacaceae	<i>Portulaca sp</i>	-	x	-		H
Apocynaceae	<i>Catharanthus</i>	<i>Catharanthus roseus</i>	x	x	MIB:SASS_0426	H

Family	Genus	Species	Vegetation quadrat	ITS2 Sequence produced in this study	Voucher name	Tree/Herbs
Moraceae	<i>Morus sp</i>	-	-	x	MIB:SASS_0427	T
Acanthaceae sp5	-	-	-	x	MIB:SASS_0429	H
Asparagaceae	<i>Chlorophytum</i>	<i>Chlorophytum sp</i>	-	x	MIB:SASS_0430	H
Asphodelaceae sp1	-	-	-	x	MIB:SASS_0432	H
Araceae	<i>Colocasia sp</i>	-	x	x	MIB:SASS_0433	H
Asteraceae sp2	-	-	x	x	MIB:SASS_0434	H
Malvaceae	<i>Sida</i>	<i>Sida pusilla</i>	x	x	MIB:SASS_0436	H
Cyperaceae	<i>Cyperus sp</i>	-	x	x	MIB:SASS_0437	H
Poaceae	<i>Setaria sp</i>	-	x	x	MIB:SASS_0439	H
Lamiaceae sp9	-	-	x	-		H
Asteraceae	<i>Erigeron sp</i>	-	x	x	MIB:SASS_0442	H
Polygonaceae	<i>Oxygonum sp</i>	-	x	x	MIB:SASS_0445	H
Asteraceae sp12	-	-	x	x	MIB:SASS_0447	H
Apiaceae	<i>Centella</i>	<i>Centella asiatica</i>	x	x	MIB:SASS_0453	H
Solanaceae	<i>Solanum</i>	<i>Solanum incanum</i>	x	-		H
Cucurbitaceae	<i>Cuscuta sp</i>	-	x	x	MIB:SASS_0454	H
Poaceae	<i>Cenchrus sp</i>	-	x	x	MIB:SASS_0456	H
Lamiaceae sp1	-	-	-	x	MIB:SASS_0457	H
Poaceae sp2	-	-	-	x	MIB:SASS_0462	H
Fabaceae	<i>Leucaena</i>	<i>Leucaena leucocephala</i>	x	x	MIB:SASS_0463	T
Acanthaceae	<i>Dyschoriste sp</i>	-	x	x	MIB:SASS_0465	H
Araliaceae	<i>Hydrocotyle</i>	<i>Hydrocotyle nepalensis</i>	x	x	MIB:SASS_0467	H
Malvaceae	<i>Corchorus</i>	<i>Corchorus olitorius</i>	x	x	MIB:SASS_0470	H
Poaceae	<i>Cynodon</i>	<i>Cynodon dactylon</i>	x	x	MIB:SASS_0473	H
Asteraceae sp13	-	-	x	x	MIB:SASS_0475	H
Caryophyllaceae	<i>Stellaria sp</i>	-	x	-		H
Fabaceae	<i>Teramnus</i>	<i>Teramnus labialis</i>	x	x	MIB:SASS_0476	H
Apiaceae sp	-	-	x	x	MIB:SASS_0477	H
Poaceae	<i>Cenchrus</i>	<i>Cenchrus purpureus</i>	x	x	MIB:SASS_0478	H

Family	Genus	Species	Vegetation quadrat	ITS2 Sequence produced in this study	Voucher name	Tree/Herbs
Convolvulaceae	<i>Convolvulus sp</i>	-	x	x	MIB:SASS_0481	H
Convolvulaceae sp2	-	-	-	x	MIB:SASS_0483	H
Poaceae sp1	-	-	x	x	MIB:SASS_0485	H
Euphorbiaceae	<i>Euphorbia</i>	<i>Euphorbia heterophylla</i>	-	x	MIB:SASS_0486	H

Table S3: List of the calculated network indices values for each of the investigated farms. Farm's identification number is the same as reported in Table S1. The network level index H2' is reported in the second column while the Foraging niche overlap, calculated as individual pollinator level index, is reported in the third column.

FARM ID	H2'	Foraging niche overlap
1	1.0000	0.2333
2	0.8561	0.1302
3	0.9336	0.1099
4	0.7038	0.1584
5	0.7650	0.1310
6	0.9602	0.1646
7	0.7835	0.2209
8	0.2635	0.1746
9	0.8483	0.2032
10	0.7881	0.1173
11	0.7916	0.0914
12	0.9272	0.3542
13	0.9807	0.2000
14	0.7780	0.1188
15	0.5953	0.2234
16	0.6575	0.1090
17	0.6799	0.1265
18	0.7257	0.0824
19	0.6588	0.0614
20	0.9168	0.0891
21	0.9396	0.1795
22	0.8193	0.1751

23	0.6934	0.3000
24	0.9072	0.1656
25	0.9069	0.0969

TABLE S4: List of the insects collected through entomological net and used to build and analyse plant-pollinator interaction networks. The first columns report to sample name, the second report the identification number of the farm where samples were collected. The last column reports the results of morphological identification, bee specimens were identified by the co-author Paolo Biella.

Sample ID	Farm ID	insect ID
B9N5	19	<i>Amegilla fallax</i>
B9N7	19	<i>Amegilla fallax</i>
B9N8	19	<i>Amegilla fallax</i>
S113	20	<i>Amegilla sp1</i>
S117	20	<i>Amegilla sp1</i>
S119	20	<i>Amegilla sp1</i>
S122	20	<i>Amegilla sp1</i>
S127	21	<i>Amegilla sp1</i>
S142	21	<i>Amegilla sp1</i>
S164	20	<i>Amegilla sp1</i>
S221	22	<i>Amegilla sp1</i>
S239	22	<i>Amegilla sp1</i>
S71	25	<i>Amegilla sp1</i>
S211	4	<i>Amegilla sp8</i>
S65	24	<i>Amegilla sp8</i>
9N2	9	<i>Anthophora cf. vestita</i>
10N1	1	<i>Apis mellifera</i>
2N7	3	<i>Apis mellifera</i>
3N7	4	<i>Apis mellifera</i>
4N2	5	<i>Apis mellifera</i>
4N4	5	<i>Apis mellifera</i>
6N1	7	<i>Apis mellifera</i>
6N2	7	<i>Apis mellifera</i>
6N5	7	<i>Apis mellifera</i>
6N8	7	<i>Apis mellifera</i>

Sample ID	Farm ID	insect ID
9N4	9	<i>Apis mellifera</i>
B11N10	10	<i>Apis mellifera</i>
B11N11	10	<i>Apis mellifera</i>
B11N12	10	<i>Apis mellifera</i>
B11N14	10	<i>Apis mellifera</i>
B11N2	10	<i>Apis mellifera</i>
B11N5	10	<i>Apis mellifera</i>
B11N8	10	<i>Apis mellifera</i>
B12N1	11	<i>Apis mellifera</i>
B12N10	11	<i>Apis mellifera</i>
B12N11	11	<i>Apis mellifera</i>
B12N16	11	<i>Apis mellifera</i>
B12N17	11	<i>Apis mellifera</i>
B12N2	11	<i>Apis mellifera</i>
B12N3	11	<i>Apis mellifera</i>
B12N4	11	<i>Apis mellifera</i>
B12N5	11	<i>Apis mellifera</i>
B12N6	11	<i>Apis mellifera</i>
B12N8	11	<i>Apis mellifera</i>
B12N9	11	<i>Apis mellifera</i>
B13N2	12	<i>Apis mellifera</i>
B13N3	12	<i>Apis mellifera</i>
B13N4	12	<i>Apis mellifera</i>
B13N5	12	<i>Apis mellifera</i>
B13N6	12	<i>Apis mellifera</i>
B14N3	13	<i>Apis mellifera</i>
B15N1	14	<i>Apis mellifera</i>
B15N6	14	<i>Apis mellifera</i>
B16N10	15	<i>Apis mellifera</i>
B16N11	15	<i>Apis mellifera</i>
B16N13	15	<i>Apis mellifera</i>
B16N14	15	<i>Apis mellifera</i>
B16N2	15	<i>Apis mellifera</i>
B16N7	15	<i>Apis mellifera</i>
B5N1	16	<i>Apis mellifera</i>
B5N10	16	<i>Apis mellifera</i>
B5N14	16	<i>Apis mellifera</i>

Sample ID	Farm ID	insect ID
B5N2	16	<i>Apis mellifera</i>
B6N1	17	<i>Apis mellifera</i>
B6N10	17	<i>Apis mellifera</i>
B6N11	17	<i>Apis mellifera</i>
B6N12	17	<i>Apis mellifera</i>
B6N13	17	<i>Apis mellifera</i>
B6N14	17	<i>Apis mellifera</i>
B6N15	17	<i>Apis mellifera</i>
B6N2	17	<i>Apis mellifera</i>
B6N3	17	<i>Apis mellifera</i>
B6N4	17	<i>Apis mellifera</i>
B6N5	17	<i>Apis mellifera</i>
B6N6	17	<i>Apis mellifera</i>
B6N7	17	<i>Apis mellifera</i>
B6N8	17	<i>Apis mellifera</i>
B6N9	17	<i>Apis mellifera</i>
B7N1	18	<i>Apis mellifera</i>
B9N14	19	<i>Apis mellifera</i>
B9N15	19	<i>Apis mellifera</i>
B9N16	19	<i>Apis mellifera</i>
S108	20	<i>Apis mellifera</i>
S109	20	<i>Apis mellifera</i>
S114	20	<i>Apis mellifera</i>
S118	20	<i>Apis mellifera</i>
S124	20	<i>Apis mellifera</i>
S128	21	<i>Apis mellifera</i>
S129	21	<i>Apis mellifera</i>
S133	21	<i>Apis mellifera</i>
S137	21	<i>Apis mellifera</i>
S144	21	<i>Apis mellifera</i>
S167	20	<i>Apis mellifera</i>
S168	20	<i>Apis mellifera</i>
S222	22	<i>Apis mellifera</i>
S230	22	<i>Apis mellifera</i>
S28	23	<i>Apis mellifera</i>
S60	24	<i>Apis mellifera</i>
S68	24	<i>Apis mellifera</i>

Sample ID	Farm ID	insect ID
S72	25	<i>Apis mellifera</i>
S76	25	<i>Apis mellifera</i>
S77	25	<i>Apis mellifera</i>
S78	25	<i>Apis mellifera</i>
S82	25	<i>Apis mellifera</i>
S84	25	<i>Apis mellifera</i>
S85	25	<i>Apis mellifera</i>
S88	25	<i>Apis mellifera</i>
S98	23	<i>Apis mellifera</i>
1AN5	2	<i>Apis mellifera</i>
2N1	3	<i>Apis mellifera</i>
3N3	4	<i>Apis mellifera</i>
B12N7	11	<i>Apis mellifera</i>
B16N9	16	<i>Apis mellifera</i>
B9N11	19	<i>Apis mellifera</i>
B9N13	19	<i>Apis mellifera</i>
B9N2	19	<i>Apis mellifera</i>
B9N9	19	<i>Apis mellifera</i>
1AN3	2	<i>Apis mellifera scutellata</i>
1AN4	2	<i>Apis mellifera scutellata</i>
1BN4	2	<i>Apis mellifera scutellata</i>
2N2	3	<i>Apis mellifera scutellata</i>
2N3	3	<i>Apis mellifera scutellata</i>
2N4	3	<i>Apis mellifera scutellata</i>
3N1	4	<i>Apis mellifera scutellata</i>
3N2	4	<i>Apis mellifera scutellata</i>
3N4	4	<i>Apis mellifera scutellata</i>
3N5	4	<i>Apis mellifera scutellata</i>
3N6	4	<i>Apis mellifera scutellata</i>
4N3	5	<i>Apis mellifera scutellata</i>
6N7	7	<i>Apis mellifera scutellata</i>
8N1	8	<i>Apis mellifera scutellata</i>
8N2	8	<i>Apis mellifera scutellata</i>
B11N13	10	<i>Apis mellifera scutellata</i>
B11N15	10	<i>Apis mellifera scutellata</i>
B11N16	10	<i>Apis mellifera scutellata</i>
B11N4	10	<i>Apis mellifera scutellata</i>

Sample ID	Farm ID	insect ID
B11N6	10	<i>Apis mellifera scutellata</i>
B11N7	10	<i>Apis mellifera scutellata</i>
B11N9	10	<i>Apis mellifera scutellata</i>
B12N12	11	<i>Apis mellifera scutellata</i>
B12N13	11	<i>Apis mellifera scutellata</i>
B12N14	11	<i>Apis mellifera scutellata</i>
B12N15	11	<i>Apis mellifera scutellata</i>
B14N1	13	<i>Apis mellifera scutellata</i>
B14N2	13	<i>Apis mellifera scutellata</i>
B14N4	13	<i>Apis mellifera scutellata</i>
B14N5	13	<i>Apis mellifera scutellata</i>
B14N6	13	<i>Apis mellifera scutellata</i>
B15N10	14	<i>Apis mellifera scutellata</i>
B15N11	14	<i>Apis mellifera scutellata</i>
B15N12	14	<i>Apis mellifera scutellata</i>
B15N13	14	<i>Apis mellifera scutellata</i>
B15N15	14	<i>Apis mellifera scutellata</i>
B15N2	14	<i>Apis mellifera scutellata</i>
B15N3	14	<i>Apis mellifera scutellata</i>
B15N4	14	<i>Apis mellifera scutellata</i>
B15N5	14	<i>Apis mellifera scutellata</i>
B15N7	14	<i>Apis mellifera scutellata</i>
B15N8	14	<i>Apis mellifera scutellata</i>
B15N9	14	<i>Apis mellifera scutellata</i>
B16N1	15	<i>Apis mellifera scutellata</i>
B16N15	15	<i>Apis mellifera scutellata</i>
B16N3	15	<i>Apis mellifera scutellata</i>
B16N4	15	<i>Apis mellifera scutellata</i>
B16N6	15	<i>Apis mellifera scutellata</i>
B5N11	16	<i>Apis mellifera scutellata</i>
B5N12	16	<i>Apis mellifera scutellata</i>
B5N13	16	<i>Apis mellifera scutellata</i>
B5N15	16	<i>Apis mellifera scutellata</i>
B5N3	16	<i>Apis mellifera scutellata</i>
B5N4	16	<i>Apis mellifera scutellata</i>
B5N6	16	<i>Apis mellifera scutellata</i>
B5N7	16	<i>Apis mellifera scutellata</i>

Sample ID	Farm ID	insect ID
B5N9	17	<i>Apis mellifera scutellata</i>
B7N14	18	<i>Apis mellifera scutellata</i>
B9N1	19	<i>Apis mellifera scutellata</i>
B9N10	19	<i>Apis mellifera scutellata</i>
B9N4	19	<i>Apis mellifera scutellata</i>
B9N6	19	<i>Apis mellifera scutellata</i>
4N5	5	<i>Bembix sp1</i>
4N6	5	<i>Betasyrphus aff. adligatus "A"</i>
8N4	8	<i>Betasyrphus aff. adligatus "A"</i>
6N4	7	<i>Ceratina moerenhouti</i>
B7N3	18	<i>Ceratina moerenhouti</i>
9N3	9	<i>Ceratina sp. 2</i>
9N7	9	<i>Ceratina sp. 2</i>
9N6	9	<i>Ceratina sp.1</i>
9N5	9	<i>Cerceris sp.1</i>
S234	22	Compsomerinae, Genus sp1
S92	23	Compsomerinae, Genus sp1
S235	22	Compsomerinae, Genus sp10
B11N3	10	<i>Episyrphus trisectus</i>
4N1	5	<i>Eristalinus cf. fuscicornis</i>
5N8	6	<i>Eristalinus myathropinus</i>
B9N12	19	<i>Eristalinus taeniops</i>
B9N3	19	<i>Eristalinus taeniops</i>
S244	4	<i>Heriades sp1</i>
10N7	1	Hymenoptera B
4N7	5	Hymenoptera D
4N9	5	Hymenoptera E
6N3	7	Hymenoptera F
8N6	8	Hymenoptera G
B7N13	18	Hymenoptera N
B7N7	18	Hymenoptera X
S209	22	<i>Lasioglossum bellulum</i>
10N2	1	<i>Lasioglossum sp.1</i>
B11N1	10	<i>Lipotriches hylaeoides</i>
B7N8	18	<i>Megachile aff ungulata</i>
B7N12	18	<i>Megachile aff. Frontalis</i>
B7N2	18	<i>Megachile aff. Frontalis</i>

Sample ID	Farm ID	insect ID
B7N5	18	<i>Megachile aff. Frontalis</i>
B7N6	18	<i>Megachile aff. Frontalis</i>
B15N14	14	<i>Megachile bituberculata</i>
2N8	3	<i>Megachile felina</i>
B7N4	18	<i>Megachile sp. 2</i>
S115	20	<i>Megachile sp16</i>
S215	22	<i>Megachile sp16</i>
S22	23	<i>Megachile sp16</i>
S238	22	<i>Megachile sp16</i>
S240	4	<i>Megachile sp16</i>
S74	25	<i>Megachile sp16</i>
S79	25	<i>Megachile sp16</i>
S89	25	<i>Megachile sp16</i>
S48	24	<i>Megachile sp16 / Brounopsis sp3</i>
S120	20	<i>Megachile sp3</i>
S83	25	<i>Megachile sp9</i>
B7N11	18	<i>Megachile venusta</i>
5N9	6	<i>Melanostoma bituberculatum</i>
9N8	9	<i>Paragus haemorrous</i>
5N7	6	<i>Paragus minutus</i>
1AN7	2	<i>Phytomia bulligera</i>
1BN2	2	<i>Phytomia bulligera</i>
1BN6	2	<i>Phytomia bulligera</i>
5N3	6	<i>Phytomia bulligera</i>
B5N5	16	<i>Phytomia bulligera</i>
1BN3	2	<i>Phytomia incisa</i>
2N6	3	<i>Phytomia incisa</i>
5N4	6	<i>Phytomia incisa</i>
1AN2	2	<i>Plebeina armata</i>
S75	25	<i>Schwaizia emmae</i>
8N3	8	<i>Seladonia foana</i>
B7N10	18	<i>Seladonia foana</i>
S229	22	<i>Seladonia foana</i>
B5N8	16	<i>Seladonia jucunda</i>
S37	23	<i>Steganomos junodi</i>
8N7	8	<i>Tetraloniella cf. alboscopacea</i>
8N8	8	<i>Tetraloniella cf. alboscopacea</i>

Sample ID	Farm ID	insect ID
9N1	9	<i>Tetraloniella cf. alboscopacea</i>
S112	20	<i>Thyreus sp3</i>
S223	22	<i>Thyreus sp4</i>
S70	24	<i>Thyreus sp4</i>
1AN6	2	<i>Toxomerus floralis</i>
1BN1	2	<i>Toxomerus floralis</i>
1BN5	2	<i>Toxomerus floralis</i>
5N1	6	<i>Toxomerus floralis</i>
5N2	6	<i>Toxomerus floralis</i>
5N5	6	<i>Toxomerus floralis</i>
5N6	6	<i>Toxomerus floralis</i>
S111	20	<i>Xylocopa flavicollis</i>
S131	21	<i>Xylocopa inconstans</i>
S163	20	<i>Xylocopa inconstans</i>
S165	21	<i>Xylocopa inconstans</i>
S206	22	<i>Xylocopa inconstans</i>
S62	24	<i>Xylocopa somalica</i>

Supplementary Text S1: Details on sample preparation, DNA extraction, sequencing, bioinformatics analysis and taxonomic assignment

After the isolation of pollen grains samples were grinded with a Tissue Lyser® II (Qiagen®, Hilden, Germany) prior to freezing in liquid nitrogen. DNA was extracted under a laminar flow cabinet using the Qiagen® DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction, with a final elution volume of 50 µl. The nuclear internal transcribed spacer 2 region (ITS2) was selected as the DNA barcode region given the high resolution shown in other studies (Chen et al., 2010; Biella et al., 2019; Frigerio et al., 2020). The selected locus was amplified using primers S2F and S3R with the addition of the Illumina overhang adapter sequences, primer sequence and sample preparation protocol prior to sequencing are extensively explained in Biella et al. (2019). Library preparation and sequencing were performed through the Illumina MiSeq instrument using MiSeq 600 V3 (2 × 300-bp paired-end sequencing). The library

preparation and the sequencing process were conducted at the Center for Translational Genomics and Bioinformatics (San Raffaele Scientific Institute, Milan, Italy). Before proceeding with the taxonomic assignment of pollen ITS2 reads, raw sequences were paired and pre-processed with QIIME2 (ver. 2019.4; <https://qiime2.org/>) (Bolyen et al., 2018). Primers were trimmed and ASVs sequences (or features) were obtained using the DADA2 algorithm (Callahan et al., 2016), performing a quality filter with an expected error of 2.0 and removing chimeras. Features outside the bounds of 200 and 550 bp were removed. The taxonomic assignment was carried out using the BLAST algorithm (Camacho et al., 2009) against our local DNA reference dataset and, in case of no reliable match, on the entire NCBI nucleotide database. After checking for the presence of unlikely matches (i.e., plants found not to be present in the study area), only assignments with a max identity and a query coverage $\geq 98\%$ were accepted. The identified species were also categorized in wild or horticultural species. The dataset generated for this study was submitted to the EBI metagenomics portal (<https://www.ebi.ac.uk/metagenomics/>). BioSamples accessions are: PRJEB41466 (ERP125246)

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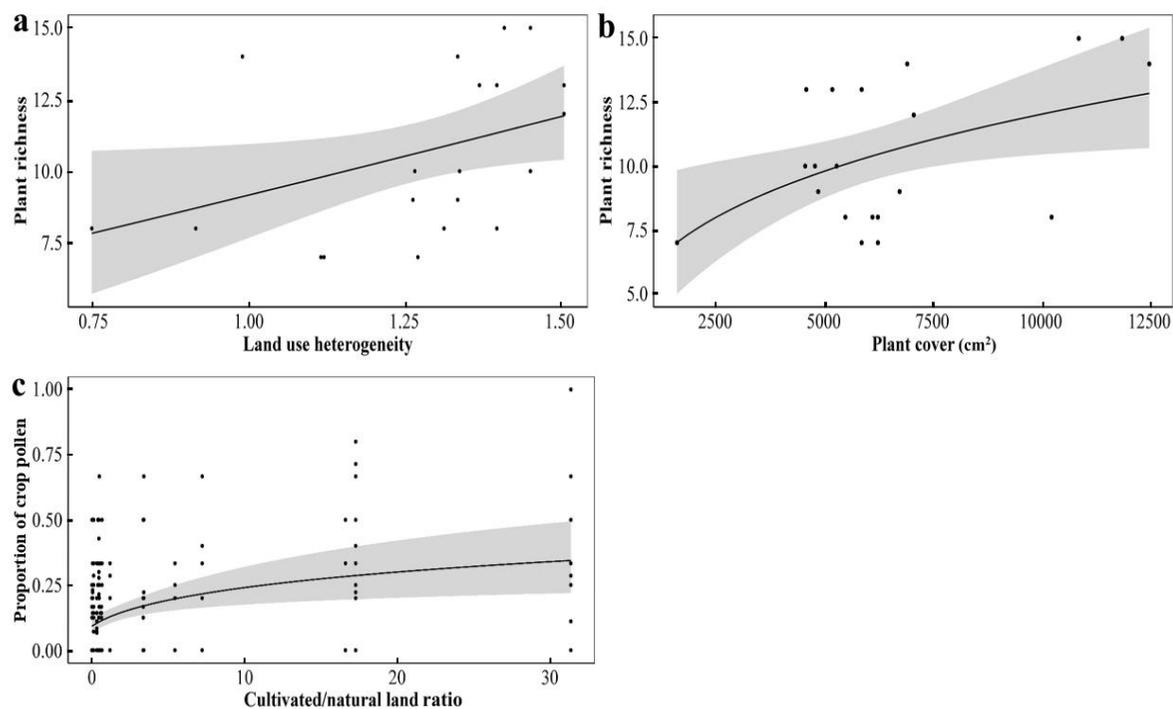
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Figure S1: Plots (a) and (b) depict the relationship between plant species and land use heterogeneity and plant cover (cm²), respectively. Plot (c) shows the result of the regression analysis of proportion of pollen from horticultural species carried by pollinators as a function of the cultivated - natural land ratio.



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8.2 Tommasi, N., Biella, P., Maggioni, D., Fallati, Agostinetto, G., L., Labra, M., Galli, P., Galimberti, A. DNA metabarcoding unveils the effects of habitat fragmentation on pollinator diversity, plant-pollinator interactions, and pollination efficiency in tropical islands. Article *submitted to "Molecular Ecology"*.

In this work the second intermediate aim of the thesis has been achieved by investigating the impact green habitat fragmentation on pollinator insects' biodiversity, interactions, and on the resulting effects on the pollination efficiency. To do this, Islands of Maldives archipelago have been used as ecological model systems due to their simplified biological communities that allows an easier and clearer comprehension of the investigated phenomena. I participated to all the phases of this experimental work, from field sampling to laboratory activities, data analysis, and article writing.

Type of Article: Research article

DNA METABARCODING UNVEILS THE EFFECTS OF HABITAT FRAGMENTATION ON POLLINATOR DIVERSITY, PLANT-POLLINATOR INTERACTIONS, AND POLLINATION EFFICIENCY IN TROPICAL ISLANDS

Running title: Plant-pollinator interactions in islands with fragmented landscapes

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ABSTRACT

Habitat fragmentation is known to affect biodiversity, but the impact on pollinators and their interactions with plants is still unclear in anthropized landscapes. Islands are open air laboratories for ecological studies with simplified communities and interactions, suitable to disentangle how land-use alteration impacts pollination ecology and its ecosystem service. Here, we used Maldives islands as model systems to investigate how pollinator richness, their mutualistic interactions with plants and pollination efficiency are shaped by the degree of green area fragmentation (i.e., gardens, parks and semi-natural green covered patches), by considering both community- and species-level responses. To do this, we surveyed pollinators from 11 islands showing a gradient of green area fragmentation. In order to characterize the interactions between plants and pollinators and obtain a novel and comprehensive view of the key ecological dynamics, a DNA metabarcoding approach was adopted to identify the pollen carried by pollinators. We found that green area fragmentation at intermediate levels played positive effects on pollinator richness. However, fragmentation decreased interaction network complexity. Intriguingly, body size mediated the effect of landscape alteration on plant-pollinator interactions, as only the largest bee species expanded the foraging breath in terms of transported pollen richness at increasing fragmentation. In parallel, the pollination efficiency increased with pollinator species richness in two sentinel plants. This study shows that moderate landscape fragmentation of green areas shapes the ecosystem service of pollination, where in spite of interactions being less complex and mediated by pollinator body size, pollinator biodiversity and potential plant reproduction are supported.

INTRODUCTION

Land-use intensification, mainly induced by the expansion of urbanization and agricultural activities, is often considered a major threat to biodiversity and specifically to pollinator species conservation (Dicks et al., 2020; Potts et al., 2010, Tommasi et al., 2021 a). This is because landscape intensification leads to habitat loss and green areas fragmentation, especially in urban environments (Kovács-Hostyánszki et al., 2017; Rathcke & Jules, 1993; Senapathi et al., 2017). As a result, pollinator community composition is impoverished by a decreased diversity of species in fragmented landscapes, as specialist pollinators easily disappear (Xiao et al., 2016). In turn, plant-pollinator interactions are expected to become more generalised, possibly due to changes in floral composition and distribution (Andrieu et al., 2009; Fortuna, and Bascompte 2006; Matthews, Cottee-Jones & Whittaker 2014). Local conditions related to floral resources (*e.g.*, flower diversity and abundance) are important drivers of pollinator community features and have previously been found to mitigate the negative impacts posed by land-use intensification both on community composition and interactions (Tommasi et al., 2021 a).

In landscapes intensively altered by human practices, green areas became of high importance for biodiversity and the effects of this fragmentation on pollinators could vary at different geographical and taxonomic scales. This translates into changes in pollination efficiency that have already been documented, albeit with idiosyncratic responses depending on the investigated species (Xiao et al., 2016). At a small scale (*i.e.*, 20 m radius), the diversity of bees appears negatively associated with the fragmentation of green areas (Hennig & Ghazoul, 2012). Conversely, at higher scales (*i.e.*, 200 or 1000 m radius), the fragmentation of green patches corresponded to increased pollinator species richness, flower visitation rates and pollination (Hennig & Ghazoul, 2012; Theodorou et al., 2020). This variability in responses to green habitat fragmentation highlights difficulties at forecasting how land-use intensification affects pollinator communities and the ecosystem service they provide. Furthermore, species can greatly diverge in their foraging strategies and contribute differently to pollination. Thus, the analysis of intraspecific variation in plant-pollinator interaction in fragmented habitats is necessary to comprehend the role of target species, and their changes in response to anthropic disturbance (Biella et al., 2019 b; Fuster & Traveset, 2020). Therefore,

it is urgent to improve our comprehension of the effects of green habitat fragmentation on pollinators to suggest ways for mitigating the impact on green ecosystems.

In this framework, islands offer unique opportunities to investigate the effects of pressures on biodiversity related to land-use (Castro-Urgal & Traveset, 2014; Kaiser-Bunbury & Blüthgen, 2015; Picanço et al., 2017; Steibl, Franke & Laforsch, 2021). Islands can be considered open air laboratories for ecological studies for several reasons. First, islands host simplified and isolated biotic communities, which allow to easily evaluate species roles in ecosystem functioning (Kaiser-Bunbury, Traveset & Hansen, 2010; Warren et al., 2015). Second, environmental changes spread earlier and more rapidly on islands than in the continent, also favored by small population sizes (Castro-Urgal & Traveset, 2014). These aspects apply also to pollinator and plant assemblages, which are usually simplified in insular ecosystems (Kaiser-Bunbury, Traveset & Hansen 2010; Traveset et al., 2016). An additional, relevant aspect is that dispersal events among islands are occasional or rare, and this is a favourable property when studying the effects that land-use changes as green areas fragmentation have on plant-pollinator interactions (Kaiser-Bunbury & Blüthgen, 2015). Therefore, islands are suitable scenarios for investigating the effects of land-use intensification on pollinator foraging and thus on their interactions with plants, which further supports the adoption of this model system to solve ecological questions.

Many insular systems are peculiar and yet largely neglected, especially in light of ecological research on terrestrial biodiversity and interactions between taxa. This is the case of Maldives, in the Indian Ocean, where studies on terrestrial biodiversity are extremely rare (Steibl, Franke, & Laforsch, 2021). In addition, studies in insular systems could be biased by poor taxonomy and species distribution knowledge. In this framework, modern molecular approaches can efficiently support investigation on species biodiversity and biological interactions. In recent years, molecular tools such as DNA metabarcoding have been increasingly applied in pollination ecology research to achieve the goal of describing plant-pollinator interactions (Bell et al., 2017; Pornon et al., 2016; Tommasi et al., 2021 a). By foraging on flowers pollinators carry pollen grains that keep trace of their foraging activity (Bosch, Martín González, Rodrigo, & Navarro 2009). Standard DNA barcode loci can be used to characterize this pollen and understand which plants were visited (Tommasi et al., 2021 b). In this way, it is

possible to reconstruct the interaction networks among plants and their pollinators, as well as to better assess the resource use preferences shown by flower visitors (Biella et al., 2019 a). This approach ensures significant advantages, allowing to reduce the time spent for field direct observation of interactions or to reduce the time spent for pollen characterization in laboratories, while improving the number of observed interactions (Bell et al., 2017). However, the potential of DNA metabarcoding for identifying pollen can be amplified when it is applied to contrasting scenarios in order to further illuminate the effects of human disturbance (Soares, Ferreira, & Lopes, 2017). This molecular information can be easily translated into network indices permitting reliable comparisons. Moreover, since flower visitation does not necessarily lead to conspecific pollen deposition (Ashman et al., 2020), the combination of DNA metabarcoding-based network analysis with measurements of pollination efficiency (*e.g.*, pollen deposition, pollen tube growth, fruit, and seed set) (Stavert, Bailey, Kirkland, & Rader, 2020) could provide a comprehensive overview of the effects of human disturbance on such ecosystem interactions.

In this study, we combined the experimental advantage of an island model with the application of DNA metabarcoding to increase our understanding on how the fragmentation of green habitats (*e.g.* green patches or parks in urbanized conditions) affects pollinator diversity, their mutualistic interaction with plants, and the resulting efficiency of the pollination service. To do so, we investigated pollinator communities in the Maldives islands, an insular context largely neglected under a pollination ecology perspective (but see Kevan, 1993). There, islands are homogeneous in terms of composition of biotic communities and geographical conditions, while varying in the degree of human exploitation and impact (Fallati, Savini, Sterlacchini, & Galli, 2017). This context results in a gradient of green area fragmentation and provides a model condition that ensures better understanding and interpretation of the impact of this fragmented landscape on pollinators, allowing knowledge transfer to other geographical contexts of landscape alteration.

Standing at the need to improve the comprehension of the effects of green habitat fragmentation on pollinator communities, here we aimed at evaluating how this phenomenon affect the ecosystem service of pollination in tropical islands by investigating several aspects: i) the pollinator species richness, ii) the

plant-pollinator interactions, considering both community and intraspecific variations, and iii) the pollination efficiency.

MATERIALS AND METHODS

Study area and landscape characterization

The study was conducted on 11 islands of the Maldivian archipelago (a number of islands comparable to those investigated by Steibl, Franke & Laforsch, 2021), located in two adjacent atolls, namely the southern part of Faafu and the northern part of Dhaalu atolls, about 150 km from the capital Male (Fig. 1). The temperature in these islands is homogeneous throughout the year, with almost no seasonal variation and a mean of 28°C (Bailey, Khalil, & Chatikavanij, 2015). The natural vegetation is characterized by dense, unfragmented coastal forests, where association of *Scaevola taccada*, *Pemphis acidula*, and *Pandanus* spp. are typically found together with coconut palm coastal plantations. Differently, the inner land of inhabited islands is characterized by scattered coconut palms along with large trees such as *Ficus benghalensis*, *Artocarpus altilis*, and several cultivated fruit or ornamental species. Exotic plant species compose almost 60% of the vegetation of the islands (Sujanapal & Sankaran, 2016). The investigated islands were selected in order to cover a gradient of land-use intensification related to anthropic activities, intended as the proportion of buildings, such as houses or infrastructures devoted to human activities. The distances among adjacent islands range between 1110 m (between islands 8 and 9, Fig 1) and 12000 m (between islands 6 and 10, Fig 1). They also differ in terms of inhabitants (0-1600 people ca, Maldives population & housing census, 2014) that leads to a gradient of land-use and habitat fragmentation across islands (Supporting Information, Fig S1). Fragmentation was estimated through the edge density index (*i.e.*, green patches edge length divided by total area), specifically calculated for the inland green patches (*i.e.*, gardens, parks and semi-natural green covered patches excluding the usually continuous coastal forest). To estimate edge density, index polygons enclosing the inland green patches have been manually drawn for each island using QGIS 3.16 and Bing Aerial base map updated to 2019. The edge density of inland green patches was calculated through the LecoS QGIS plugin (Jung, 2013). In the five biggest islands (*i.e.*, those with a major axis greater than 500 m) two

sampling sites were selected. Overall, 17 sampling sites were included in the survey (Supporting information -Table S1).

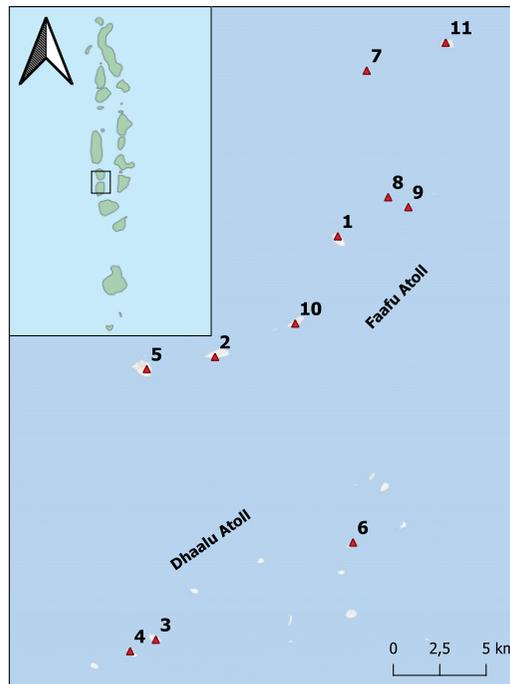


Fig1: Localization of the 11 islands of the Faafu (North) and Dhaalu (South) atolls. Island full names and coordinates are provided in Supporting information, Table S1. The gradient of green habitat fragmentation is reported in Supporting Information, Fig S1. This map was created using QGIS version 3.16.10 (<https://www.qgis.org/it/site/>).

Pollinator insects and plant characterization

Sampling activities were performed in October 2019, from 9:00 to 16:00 only with good climate conditions. Pollinator insects were sampled by hand-collecting flower visitors through entomological nets along free transects around and crossing over an area of continuous vegetation about 50 m x 50 m at each sampling location. Pollinator insects were captured after being observed foraging on flowers for a few seconds and captures were performed during a fixed time of three hours to allow unbiased comparison between sampling sites (similarly to Tommasi et al., 2021 a). After collection, each insect was individually stored in a clean tube and filled with 70% ethanol. A clean entomological net was used in

each sampling site to prevent cross contamination between samples from different sites.

Insects identification was performed through morphological inspection and via standard DNA barcoding (Supporting information - Table S2). Specifically, insects were first sorted at the lowest possible taxonomic level (family or genus) following morphological criteria and identification keys (*e.g.*, Bratra, 1977; Gupta, 2003; Thompson, 1981). Morpho-species grouping was subsequently confirmed by analysing representative individuals (1- 11 individuals representative for sex and atoll of provenance) for each morpho-species through a standard DNA barcoding approach using primers LCO1490 and HCO2198 (Folmer et al., 1994) and the laboratory protocols described in Galimberti et al., 2021. For doubtful identifications where two or more candidate DNA barcode identification were equally likely, a detailed morphological scrutiny was additionally performed by an expert (author PB). Flower species richness was also estimated identifying all the flowering species observed in the whole area involved in insect sampling and plants were identified in the field or in the lab following Sujanalpal & Sankaran (2016).

Sampling localities and details are available in BOLD under the project code ZPLML https://www.boldsystems.org/index.php/MAS_Management_DataConsole?codes=ZPLML and are also reported in Supporting information - Table S1.

Pollen DNA metabarcoding and plant-pollinator networks

The taxonomic composition of the pollen carried on insects' bodies was used to retrieve information about their interactions with plants following a procedure similar to Tommasi et al (2021 a). Briefly, pollen was recovered from insects by vortexing the tubes containing insects and ethanol for at least 10 s. Insects were removed from the ethanol and tubes were centrifuged at 14000 rpm for 10 minutes. Afterwards, the ethanol was removed through evaporation in a chemical hood. Pollen was grinded through a Tissue Lyser[®] (Qiagen) after being frozen in liquid nitrogen, then DNA was extracted following DNeasy Plant mini kit[®] (Qiagen) according to manufacturer's protocol. Negative controls were also produced for each day of laboratory activity during the DNA isolation phase. Primers S2F and S3R (Chen et al., 2010) were used with the addition of the Illumina overhang sequence adapters to amplify the internal transcribed spacer 2

(ITS2) region. Illumina standard protocol was used for Library preparation and sequencing was performed through Illumina Miseq 600 V3 (2 × 300-bp paired-end sequencing). Raw sequencing reads were paired using QIIME2 (ver. 2019.4; <https://qiime2.org/>) (Bolyen et al., 2019). After primer trimming, removal of chimera and low-quality reads, a 0.97 clustering was performed, keeping only features between 200 and 500bp (Torbjørn et al., 2016).

DNA reference sequences of the plant species identified at the study sites but not available in NCBI GenBank, were obtained by collecting leaf samples and sequencing the ITS2 region as described in Tommasi et al. (2021 a) (details on the produced DNA sequences are available in Supporting information, Table S3). Reads taxonomic assignments were carried out using the BLAST algorithm (Camacho et al., 2009) on the NCBI nucleotide database and on the local database including the reference sequences we produced. Only assignments with a max identity and a query coverage $\geq 98\%$ were accepted. Species occurrences were filtered by removing implausible matches (i.e. plants found not to be present in the study area), and by removing those represented by a reads count lower than the maximum number of reads (i.e 24 reads) produced by negative controls (Tommasi et al., 2021 b).

The taxonomic identification of pollen samples was used to retrieve interactions between plants and pollinators. First, the interaction matrix between pollinator insects and plants obtained from DNA metabarcoding was used to calculate the network index of Connectance, through the R package *Bipartite* (Dormann, Gruber, & Fründ, 2008). This, calculated as the number of actually observed interactions divided by the number of the possible interactions (Biella, Ollerton, Barcella & Assini, 2017), provides an overall estimation of network specialization and complexity. Furthermore, to evaluate intraspecific changes in the foraging strategies in response to habitat fragmentation, we selected three pollinator species belonging to three different body size classes (estimated measuring intertegular (IT) distance of thirty individuals per species) and that were uniformly distributed across islands: *Braunsapis picitarsis*. (small, mean IT 1.3 mm), *Lasioglossum albescens* (intermediate, mean 1.6 mm), and *Xylocopa fenestrata* (large, mean 7.1 mm). Specifically, the number of plant taxa found in pollen samples retrieved from each individual pollinator (individual degree) was calculated.

Pollination efficiency

Pollination efficiency was estimated by counting pollen tubes on pistil. This approach is commonly employed for this purpose since it provides a good proxy of conspecific pollen deposition (Biella et al., 2019 b, Akter, Biella, Batáry, & Klečka, 2020) and seed production (Alonso, Herrera, & Ashman, 2012). Twenty pistils of the more abundant flowering species were collected at each sampling site, stored in a solution of EtOH and CH₂O (66.5/33.5/2 EtOH/H₂O/CH₂O v/v/v) and preserved at room temperature until further analysis. Once in laboratory, pistils were softened in 4 M NaOH and stained with 0.1% aniline blue in 0.1 M K₂HPO₄ for 12 hours. After being washed with distilled water, pistils were mounted on slides with glycerine and observed with a fluorescence microscope. When pollen tubes were not visible, we counted the number of pollen grains on stigmas considering that only pollen with tubes still attached to the stigma should remain after sample preparation (Biella et al., 2019 b) and that this number is expected to be correlated to the amount of germinated tubes (Stavert, Bailey, Kirkland, & Rader 2020). Pollen tube counts were performed for those species uniformly distributed among sampling sites, specifically *Tridax procumbens*, *Wollastonia biflora*, and *Scaevola taccada*.

Statistical analysis

Changes in pollinator species richness were evaluated in response to green area fragmentation (*i.e.*, the variable Edge Density) and flower richness (*i.e.*, the number of flowering species per site). To do this, a Generalised linear mixed model (GLMM) regression (glimmTMB R package; Magnusson et al., 2017) with Poisson distribution was used, with island included as a random effect. The flower richness was included as a predictor along with the edge density, since it could represent an important local driver of pollinator richness (Blüthgen & Klein 2011). The same variables (edge density, and flower richness) were used along with the network size as predictors of change of the Connectance network index. Network size, calculated as the product between the number of insects and plants included in the networks for each site, was also included as a predictor in the model to account for its effect on Connectance variation (as in Biella et al., 2020). In this case, a GLMM with beta distribution and island included as a random effect was

used.

Changes in individual pollinator degree were evaluated in response to green area fragmentation and flower richness. The effects of these covariates were evaluated in interaction with the pollinator species identity to highlight differences among the considered pollinator species. A GLMM with Poisson distribution was used, with sites nested in the island as a random effect.

Variation in the pollination efficiency was evaluated using the pollinator richness and the Connectance as covariates. Moreover, the plant degree (mean number of pollinator species interacting with each plant species considered in pollination efficiency analysis) was calculated from DNA metabarcoding data to estimate the mean plant generalism and included as model covariate. The role of these covariates was evaluated in interaction with the plant species identity, to highlight differences among the investigated plants. A GLMM with negative binomial distribution was used to account for overdispersion. Also in this case, the site nested in island was included as a random effect.

All the analyses were performed with R (version 3.6.1; R CoreTeam 2019). Predictor significance was evaluated through a log likelihood ratio test ($P < 0.05$). The Vif function of the car package (*i.e.*, Variance Inflation Factor with an exclusion threshold of 3 (Zuur, Ieno, & Smith, 2007)) was used to exclude collinearity among variables. In all cases, the final models were obtained by removing the variables that did not improve the model fit through backward stepwise regression based on second-order Akaike Information Criterion (AIC) (Zuur et al., 2009) calculated with the package MuMIn.

RESULTS

Pollinator and plant communities

The field survey yielded 333 pollinator insects belonging to 25 morpho-species. Specifically, 72.4% of the individuals were Anthophila bees, 12.% wasps, 10.2% Syrphidae, and 5.4% were Bombyliidae flies. These were identified with DNA barcoding, in Supporting information, Table S2 - List of collected pollinator species and DNA barcode sequence. The most represented and widely distributed taxa were *Lasioglossum albescens* with 79 individuals, followed by *Braunsapis picitarsis* with 53 individuals and *Xylocopa fenestrata* with 42 individuals. Considering plants, 48 flowering species were observed in the sampling sites during the survey of flower species richness, with a minimum of 3 and maximum of 14 flowering

species present in each site. The most widespread species among sampling sites were *Tridax procumbens*, *Cyanthillium sp.*, *Scaevola taccada*, and *Wollastonia biflora*. Details on the flowering plants observed in sampling sites are reported in Supporting information, Table S3. Concerning the effects of habitat fragmentation on pollinator communities (Table 1), the edge density had a significant, positive effect in increasing pollinator species richness (Fig 2 a), while no significant effects were found in response to flower species richness.

Pollen DNA metabarcoding and plant-pollinator interactions

Pollen DNA metabarcoding yielded interactions between the collected insects and 112 plant taxa. Among these, 81 (72.3%) were assigned at species level, 28 (25%) at genus level, and 3 (2.7%) at family level. Plant taxa found in the overall pollen transported by insects on each island ranged between 9 and 58 (mean 26.4 ± 16.7), while the mean number of pollen plant taxa transported by each sampled insect was 3.5 ± 2.1 . In plant-pollinator networks constructed from the DNA metabarcoding data, the Connectance index resulted significantly and negatively associated with the Edge density (Fig 2 b) and network size (Table 1). Individual pollinator degree resulted significantly, positively related to the Edge density. However, the responses of different insect species among the widespread and most abundant ones were not homogeneous in this case, as the values of this index significantly increased in the biggest bee *Xylocopa fenestrata* ($\beta_i = 14.9$; $p = 0.001$), while it was not significant in the case of the two smaller bees: *Lasioglossum albescens* ($\beta_i = 2.94$; $p = 0.52$) and *Braunsapis picitarsis* ($\beta_i = -6.02$; $p = 0.24$) (Fig 3).

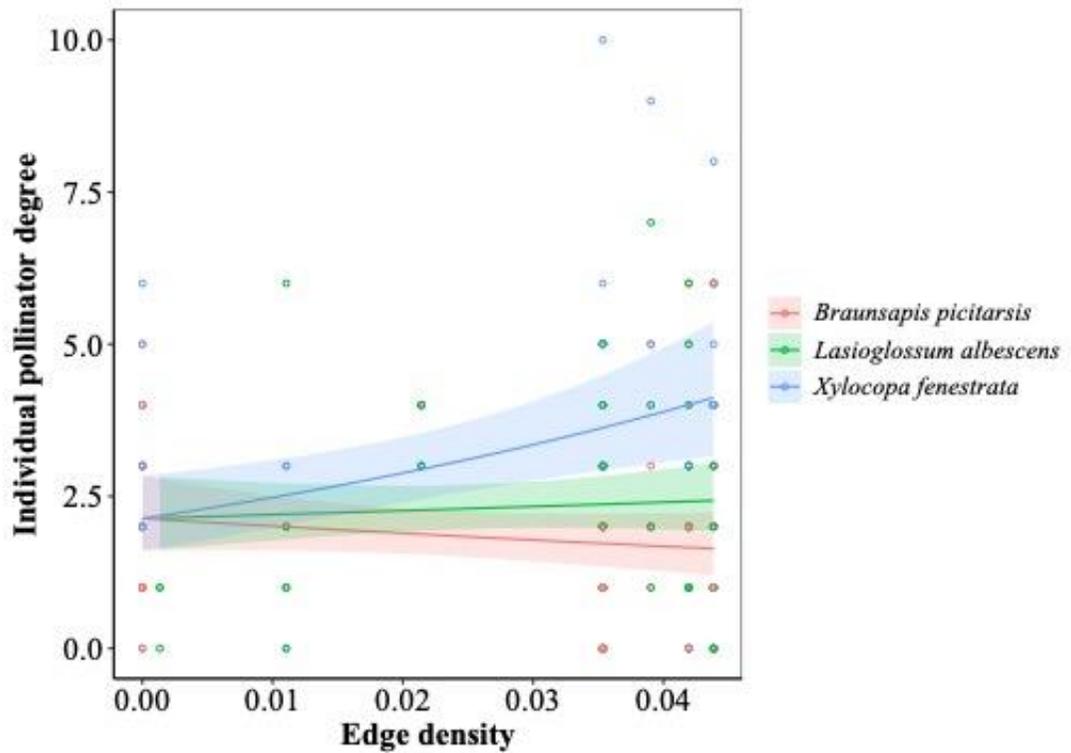


Fig 3: Individual pollinator degree, from the number of pollen species carried on the pollinator body, as a function of Edge density, a measure of habitat fragmentation, for three pollinator species of different body size. The coloured lines and areas respectively indicate the relationship and its confidence intervals as estimated with Generalised linear mixed models.

Table 1: Output of different Generalised linear mixed models of pollinator species richness, Connectance, individual pollinator degree. Final models were selected through backward stepwise selection using AIC criterion. Δ AIC reports the difference in AIC values between full and final models. β_i : regression coefficient of a given variable; χ^2 : chi square values; df: degrees of freedom. P value stands for statistical significance after log likelihood ratio test, and it is in bold if below 0.05. The regression coefficients for each of the three pollinator species considered in interaction with the predictors in the individual pollinator degree model correspond to a - *Xylocopa fenestrata*, b - *Lasioglossum albescens*, and c - *Braunsapis picitarsis*.

Response variable	Initial model covariates	Final model covariates	Δ AIC	B_i	χ^2 ; df	p value
Pollinator richness	Edge density	Edge density	2.49	16.5	11.01; 1	0.02
	Flower richness					
Connectance	Edge density	Edge density	0	-15.5	4.03; 1	0.04
	Flower richness	Flower richness		0.05	3.57; 1	0.06
	Network size	Network size		-0.3	7.95; 1	<0.001
Individual pollinator degree	Edge density x (pollinator species)	Edge density x pollinator species	4.28	a = 14.9 b = 2.94 c = -6.02	27.63; 3	<0.001
	Flower richness x (pollinator species)					

Pollination efficiency

Overall, 242 pistils were analysed, 96 for *Wollastonia biflora*, 77 for *Tridax procumbens*, and 69 for *Scaevola taccada*. Only the interaction between local pollinator richness and plant species identity resulted significantly and positively associated with the number of pollen tubes counted on stigmas ($\chi^2 = 40.37$; $p < 0.001$), with different trends among the investigated species as highlighted in Fig 4. Specifically, pollination efficiency increased in *Wollastonia biflora* ($\beta_i = 0.19$; $p = 0.001$), and *Scaevola taccada* ($\beta_i = 0.19$; $p = 0.002$), while it was constant in *Tridax procumbens* ($\beta_i = -0.02$; $p = 0.7$).

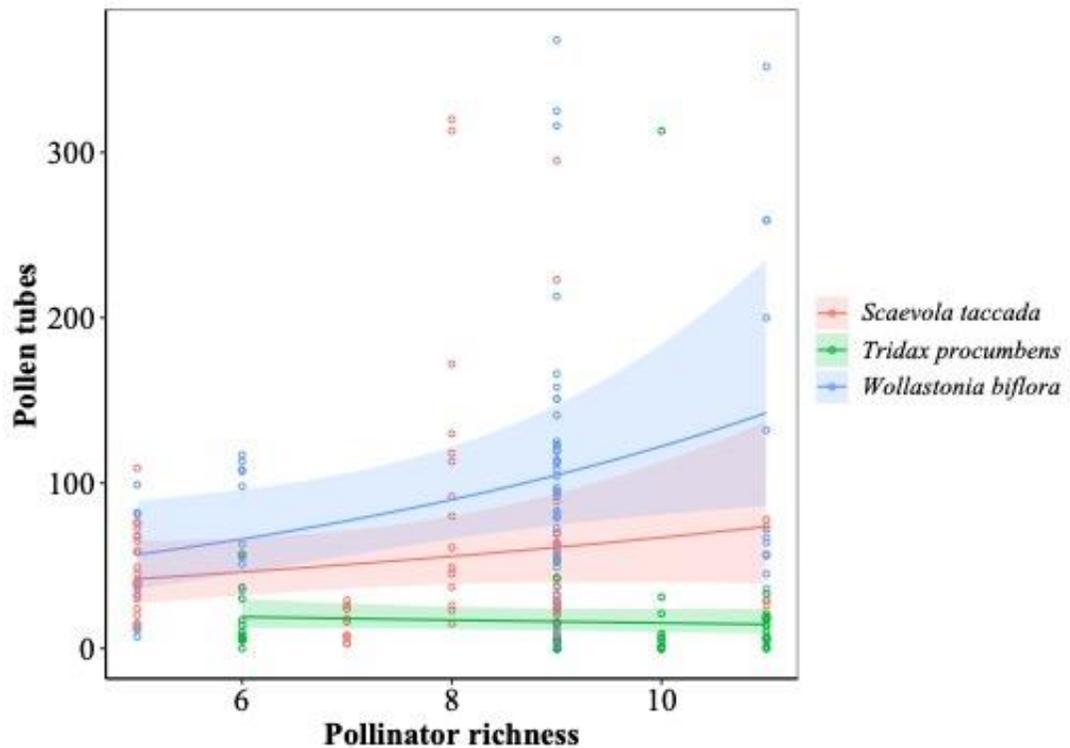


Fig 4: Regression analysis of the number of pollen tubes on stigmas as a function of environmental pollinator species richness. The coloured lines and areas respectively indicate the estimated relationship and its confidence intervals with Generalised linear mixed models, see methods for further details.

DISCUSSION

In this study, a DNA metabarcoding approach to describe plant-pollinator interactions has been coupled with pollinator traits and deposited pollen data to disentangle the effects of habitat fragmentation in an oceanic insular system. In this way, we described the direct effects of green areas fragmentation at several levels, ranging from pollinator species richness to their interactions with plants and the pollination efficiency. Alongside, we provided a characterization of the flower-visiting insects (*i.e.*, Hymenoptera and Diptera) of Maldives islands and the associated flora, that to the best of our knowledge were largely unexplored in this biodiversity hotspot.

Green areas fragmentation increased pollinator species richness. This result is consistent with the observations from other studies at comparable spatial scales.

For instance, Theodorou et al. (2020), found that bee richness increased with edge density of green spaces both in urban and rural landscapes. A similar positive trend in species richness was observed elsewhere at levels of disturbance comparable to those in our study (Kremen, 2005; Rader, Bartomeus, Tylianakis & Laliberté, 2014). However, it seems that other guilds, such as soils macroinvertebrates, have been found to decrease with fragmentation in oceanic islands (Steibl, Franke & Laforsch, 2021). Nevertheless, differences between pollinator and macroinvertebrate responses could be searched in different natural histories of these groups. Given the intermediate levels of Edge density on Maldives Islands (range 0.00 - 0.04) compared to continental levels, it seems appropriate to explain our results to the Intermediate Disturbance Hypothesis according to which species diversity is expected to increase when the disturbance is of moderate entity (Huston, 2014; Rutgers-Kelly, 2005). This may occur as a trade-off between the competitive exclusion that characterizes the absence of disturbance and the abiotic limitation found in highly disturbed conditions (Lazarina et al., 2019; Lazaro et al., 2016). Moreover, an intermediate disturbance could potentially promote foraging and nesting heterogeneity of pollinator insects, thus permitting to host more species and guarantee their survival and reproduction (Wenzel, Grass, Belavadi, & Tschardtke, 2020; Winfree et al., 2009). Our interpretation is further supported because the maximum fragmentation in our study system corresponded to a proportion of surfaces occupied by infrastructures of about thirty percent (see Supporting Information, Fig S2), that still represents an intermediate degree of disturbance (Wenzel, Grass, Belavadi, & Tschardtke, 2020). At higher disturbances, the pollinator richness is supposed to decrease, as observed by Rader et al. (2014).

Our study found that green area fragmentation clearly shapes plant-pollinator interactions both at the community and individual levels. We observed a decrease in Connectance, indicating a lowered proportion of realized interactions, that highlights a simplification of plant-pollinator networks. As Connectance is often related to network complexity and stability (May, 1972), it is likely to interpret that increasing fragmentation will lead to impoverished, more simplified networks. We understand this as an alarming aspect as a high complexity could mean high functional redundancy and it is a desirable property of functioning ecosystems (Kaiser-Bunbury et al., 2017). In spite of the effects of fragmentation at the community level, idiosyncrasies emerged when considering the interactions

of pollinators of three different body sizes. The largest among these bees, *Xylocopa fenestrata*, was the only one that modified, and specifically increased, the number of foraged plant species in response to increased fragmentation, as indicated by DNA metabarcoding of pollen. Conversely, smaller species did not show expansions or contractions of the number of foraged plant species. Differential responses depending on body size offer the key to interpret the effects of fragmentation, since size is a functional trait related to flight range (Greenleaf, Williams, Winfree, & Kremen, 2007). In fact, small pollinators usually forage in smaller patches and might even benefit from having small habitat fragments (Tscharntke, Steffan-Dewenter, Kruess & Thies, 2002) and they likely did not suffer from the level of fragmentation in oceanic islands. Differently, a larger species flying across fragments could acquire more flower resources and this is what we observed in *X. fenestrata*. Species responding in different ways to land-use change were already observed, according to their functional traits (Wenzel, Grass, Belavadi, & Tscharntke, 2020). Therefore, it is a priority to couple community trends and the responses of single species, as they are highly important to preserve community structure and functionality for instance by establishing new interactions after a disturbance event (e.g., Biella et al., 2020). The effects on the ecosystem service of pollination by green area fragmentation were evaluated by quantifying pollination efficiency in a panel of widely distributed plant species used here as sentinel cases. Specifically, pollination efficiency associated with pollinator species richness at the sites, also related to green area fragmentation. This agrees with an increasing amount of evidence supporting a positive relationship between pollinator richness and plant reproduction (Fontaine, Dajoz, Meriguet, & Loreau, 2006; Garibaldi, Steffan-Dewenter & Winfree, 2013; Mallinger & Gratton, 2015; but see Biella et al., 2021). Furthermore, pollinator species richness in an area may increase pollination efficiency through other mechanisms such as facilitation (the interactions between different species affect their foraging behaviours enhancing the pollen deposition), and temporal and/or spatial complementarity (Mallinger & Gratton, 2015). However, in our dataset, the positive trend of pollination efficiency with environmental pollinator richness was independent to the amount of pollinator species visiting a given plant, a measure of plant interaction generalism. This suggests that not all plants respond in a similar way to pollinator availability (see Biella et al., 2019 b). Overall, these results highlight the importance of

safeguarding pollinator biodiversity for maintaining the equilibrium of pollination service.

CONCLUSIONS

This study represents a valuable case of integrating field observations, laboratory approaches and molecular tools for species and interaction identification. An additional relevance is given by the study system, which comprises small oceanic islands offering unique conditions to investigate direct ecological issues in isolated conditions with limited dispersal of individuals elsewhere. Moreover, the presence of usually simplified island communities makes it easier to quantify effects and causes of land-uses on indigenous biodiversity (Picanço et al., 2017; Jupiter, Mangubhai & Kingsford 2014). Overall, these features make small oceanic islands efficient model systems to comprehend the multi-level impacts of green area fragmentation. These could offer the possibility to transfer the obtained results to mainland contexts threatened by human activities and then to address proper mitigation solutions or to predict the effects of land-use alterations.

In the Maldives the rapid development of tourism and human settlement expansion represent the main drivers of change in the landscape composition and configuration (Fallati, Savini, Sterlacchini, & Galli, 2017). These changes highlight the need to find and promote suitable solutions to support biodiversity and ecological functioning. Indeed, we proved that a moderate green area fragmentation could even promote the biodiversity of pollinators, suggesting the need to assess tolerable disturbance thresholds in specific environmental contexts to develop local land-use planning aimed at promoting pollinator biodiversity. Moreover, we stress for a higher mitigation of harmful land-uses and favouring pollinator friendly interventions (e.g., promoting floral resources and availability of heterogeneous nesting sites). This would increase pollination success and thus improve the efficiency of the pollination service. Furthermore, as species-specific responses often take place in spite of community-averaged trends, the heterogeneity of pollinator life histories should be considered when choosing how to mitigate the effect of landscape alteration. In this view, the implementation of modern molecular tools such as DNA metabarcoding in ecological studies is of growing interest in the study of mutualistic interactions. As demonstrated by this study, it provides suitable information that can be easily integrated with field data

to improve the efficiency of monitoring programs even in those contexts largely neglected by ecological research.

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DATA ACCESSIBILITY

The DNA metabarcoding data generated for this study are available on EBI metagenomics portal (<https://www.ebi.ac.uk/metagenomics/>) study ID: PRJEB49045. Plants DNA barcoding sequences produced are available in GenBank under accession numbers OL672899-OL672949. Pollinator DNA barcode sequences produced are available in BOLD system under the project code ZPLML [Accession number of insects sequences will be released upon paper acceptance].

BENEFIT-SHARING

Benefits from this research accrue from the sharing of our data and results on public databases as described above

AUTHOR CONTRIBUTIONS

Conceptualization, NT, PB and AG; Investigation, NT, PB, DM, LF; Formal Analysis, NT, PB, GA; Writing - Original Draft, NT; Writing - Review and Editing, NT, PB, AG, DM, LF, AG, GA ; Funding acquisition, PG, AG.

Supporting Information

Table S1 - Sampling site location

ISLAND ID	SITE ID	LATITUDE	LONGITUDE	ISLAND NAME	ATOLL
1	1A	3.11997	72.98391	Bileiydhoo	Faafu
1	2A	3.11535	72.98645	Bileiydhoo	Faafu
2	1B	3.0613	72.92438	Dharanboodhoo	Faafu
2	2B	3.06326	72.93006	Dharanboodhoo	Faafu
3	1C	2.92381	72.89568	Rinbudhoo	Dhaalu
3	2C	2.92525	72.89421	Rinbudhoo	Dhaalu
4	1D	2.91822	72.88323	Aavee	Dhaalu
5	1E	3.05537	72.89129	Nilandhoo	Faafu
5	2E	3.05675	72.8866	Nilandhoo	Faafu
6	1F	2.97111	72.99134	Udhdhoo	Dhaalu
7	1G	3.20051	72.99795	Jinnathugau	Faafu
8	1H	3.138977	73.00835	Adanga	Faafu
9	1N	3.13416	73.01814	Enbulufushi	Faafu
10	1L	3.07754	72.9633	Magoodhoo	Faafu
10	2L	3.08023	72.96583	Magoodhoo	Faafu
10	3L	3.07754	72.9633	Magoodhoo	Faafu
11	1M	3.21412	73.03626	Filitheyo	Faafu

Fig S1: The histogram shows the gradient of green habitat fragmentation (Green patches Edge density) across the investigated islands. Island identification numbers are reported in Table S1

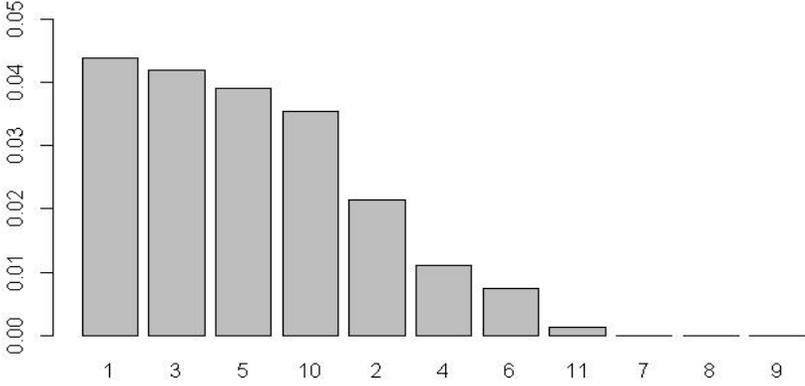


Fig S2: The histogram shows the gradient of surface proportion covered by infrastructures across the investigated islands.

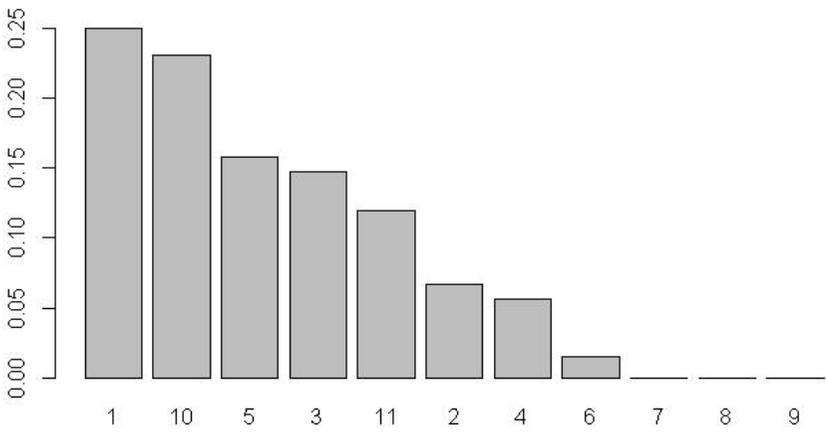


Table S2 - List of collected pollinator species. Table reporting details on insect identification. The number of individuals involved in DNA barcoding identification is reported in the column “N° of DNA barcoded specimens”. In the last column it is reported the accession number of the sequences deposited in BOLD.

Order	Family	Subfamily	Genus	Species	N° of DNA barcoded specimens	Accession number
Diptera	Syrphidae	Syrphinae	Allobaccha	<i>Allobaccha</i> sp1	2	TO BE RELEASED
Hymenoptera	Vespidae	Eumeninae	Allorhynchium	<i>Allorhynchium metallicum</i>	1	TO BE RELEASED
Hymenoptera	Bembicidae	Bembicinae	Bembecinus	<i>Bembecinus pusillus</i>	1	TO BE RELEASED
Hymenoptera	Bembicidae	Bembicinae	Bembix	<i>Bembix oculata</i>	5	TO BE RELEASED
Diptera	Bombyliidae			<i>Bombyliidae</i> sp1	11	TO BE RELEASED
Hymenoptera	Apidae	Xylocopinae	Braunsapis	<i>Braunsapis picitarsis</i>	13	TO BE RELEASED
Hymenoptera	Scoliidae	Campsomerinae	Campsomeriella	<i>Campsomeriella</i> sp1	9	TO BE RELEASED
Hymenoptera	Apidae	Xylocopinae	Ceratina	<i>Ceratina binghami</i>	10	TO BE RELEASED
Hymenoptera	Sphecidae	Sceliphrinae	Chalybion	<i>Chalybion japonicum</i>	1	TO BE RELEASED
Diptera	Syrphidae	Eristalinae	Eristalinus	<i>Eristalinus</i> sp1	5	TO BE RELEASED
Hymenoptera	Halictidae			<i>Halictidae</i> sp1	3	TO BE RELEASED
Hymenoptera	Halictidae	Halictinae	Lasioglossum	<i>Lasioglossum albescens</i>	24	TO BE RELEASED
Hymenoptera	Megachilidae	Lithurginae	Lithurgus	<i>Lithurgus</i> sp1	1	TO BE RELEASED
Hymenoptera	Megachilidae	Megachilinae	Megachile	<i>Megachile laticeps</i>	4	TO BE RELEASED
Diptera	Syrphidae	Syrphinae	Paragus	<i>Paragus serratus</i>	3	TO BE RELEASED
Hymenoptera	Vespidae	Polistinae	Polistes	<i>Polistes olivaceus</i>	1	TO BE RELEASED
Hymenoptera	Vespidae	Polistinae	Polistes	<i>Polistes stigma</i>	1	TO BE RELEASED
Hymenoptera	Vespidae	Eumeninae	Subancistrocerus	<i>Subancistrocerus sichelii</i>	1	TO BE RELEASED
Diptera	Syrphidae	Eristalinae	Syritta	<i>Syritta</i> sp1	7	TO BE RELEASED

Order	Family	Subfamily	Genus	Species	N°of DNA barcoded specimens	Accession number
Diptera	Syrphidae	Eristalinae	Syritta	<i>Syritta</i> sp2	2	TO BE RELEASED
Hymenoptera	Apidae	Xylocopinae	Xylocopa	<i>Xylocopa bryorum</i>	3	TO BE RELEASED
Hymenoptera	Apidae	Xylocopinae	Xylocopa	<i>Xylocopa fenestrata</i>	4	TO BE RELEASED
Hymenoptera	Apidae	Xylocopinae	Xylocopa	<i>Xylocopa tenuiscapa</i>	2	TO BE RELEASED

Table S3- List of identified plant species and production of DNA barcode sequence. The table reports all the plant species found on the investigated islands. The column “ITS2 sequence produced” shows the species for which a DNA barcode reference sequence was not available in GenBank and has been produced in this study. The column “Accession number” indicates the identification code of the ITS2 sequence deposited on GenBank.

Family	Genus	species	ITS2 sequence produced	Accession number
Malvaceae	Abutilon	<i>Abutilon indicum</i>		
Amaranthaceae	Aerva	<i>Aerva congesta</i>	x	OL672908
Amaranthaceae	Aerva	<i>Aerva lanata</i>	x	OL672903
Moraceae	Artocarpus	<i>Artocarpus altilis</i>	x	OL672907
Oxalidaceae	Averrhoa	<i>Averrhoa bilimbi</i>	x	OL672924
Oxalidaceae	Averrhoa	<i>Averrhoa sp</i>	x	OL672925

Family	Genus	species	ITS2 sequence produced	Accession number
Nyctaginaceae	Boerhavia	<i>Boerhavia diffusa</i>	x	OL672935
Nyctaginaceae	Boerhavia	<i>Boerhavia erecta</i>	x	OL672940
Campanulaceae	Campanula	<i>Campanula viola</i>		
Fabaceae	Canavalia	<i>Canavalia cathartica</i>	x	OL672946
Solanaceae	Capsicum	<i>Capsicum sp</i>	x	OL672948
Apocynaceae	Catharanthus	<i>Catharanthus roseus</i>	x	OL672905
Cleomaceae	Cleome	<i>Cleome viscosa</i>	x	OL672923
Lamiaceae	Clerodendrum	<i>Clerodendrum inerme</i>		
Fabaceae	Clitoria	<i>Clitoria ternatea</i>	x	OL672906 - OL672930
Malvaceae	Corchorus	<i>Corchorus aestuans</i>		
Boraginaceae	Cordia	<i>Cordia subcordata</i>	x	OL672922
Amaryllidaceae	Crinum	<i>Crinum asiaticum</i>		
Cucurbitaceae	Cucurbita	<i>Cucurbita sp</i>		

Family	Genus	species	ITS2 sequence produced	Accession number
Asteraceae	Cyanthillium	<i>Cyanthillium sp</i>		
Asteraceae	Emilia	<i>Emilia sonchifolia</i>	x	OL672917
Euphorbiaceae	Euphorbia	<i>Euphorbia cyathophora</i>		
Euphorbiaceae	Euphorbia	<i>Euphorbia hirta</i>		
Euphorbiaceae	Euphorbia	<i>Euphorbia sp</i>		
Euphorbiaceae	Euphorbia	<i>Euphorbia splendens</i>		
Euphorbiaceae	Euphorbia	<i>Euphorbia superba</i>		
Convolvulaceae	Evolvulus	<i>Evolvulus alsinoides</i>	x	OL672944
Colchicaceae	Gloriosa	<i>Gloriosa superba</i>		
Oleaceae	Guettarda	<i>Guettarda speciosa</i>	x	OL672931
Rubiaceae	Hedyotis	<i>Hedyotis sp</i>	x	OL672902
Malvaceae	Hibiscus	<i>Hibiscus sp</i>	x	OL672941
Convolvulaceae	Ipomoea	<i>Ipomoea pes-caprae</i>	x	OL672910

Family	Genus	species	ITS2 sequence produced	Accession number
Convolvulaceae	Ipomoea	<i>Ipomoea quamoclit</i>	x	OL672938
Convolvulaceae	Ipomoea	<i>Ipomoea sp</i>	x	OL672909
Rubiaceae	Ixora	<i>Ixora sp</i>	x	OL672926 - OL672927
Oleaceae	Jasminum	<i>Jasminum multiflorum</i>	x	OL672901
Verbenaceae	Lantana	<i>Lantana camara</i>		
Asteraceae	Launaea	<i>Launaea sarmentosa</i>		
Cucurbitaceae	Luffa	<i>Luffa cylindrica</i>	x	OL672934
Nyctaginaceae	Mirabilis	<i>Mirabilis jalapa</i>	x	OL672915
Rubiaceae	Morinda	<i>Morinda citrifolia</i>		
Muntingiaceae	Muntingia	<i>Muntingia calabura</i>		
Rubiaceae	Oldenlandia	<i>Oldenlandia sp</i>	x	OL672919
Passifloraceae	Passiflora	<i>Passiflora edulis</i>	x	OL672945
Passifloraceae	Passiflora	<i>Passiflora suberosa</i>	x	OL672914

Family	Genus	species	ITS2 sequence produced	Accession number
Lythraceae	Pemphis	<i>Pemphis acidula</i>		
Verbenaceae	Phyla	<i>Phyla nodiflora</i>	x	OL672913
Phyllanthaceae	Phyllanthus	<i>Phyllanthus amarus</i>	x	OL672937
Solanaceae	Physalis	<i>Physalis peruviana</i>		
Apocynaceae	Plumeria	<i>Plumeria pudica</i>	x	OL672904
Apocinaceae	Plumeria	<i>Plumeria sp</i>		
Portulacaceae	Portulaca	<i>Portulaca sp</i>		
Euphorbiaceae	Ricinus	<i>Ricinus communis</i>		
Acanthaceae	Ruellia	<i>Ruellia tuberosa</i>	x	OL672929 - OL672939
Goodeniaceae	Scaevola	<i>Scaevola taccada</i>		
Fabaceae	Senna	<i>Senna sp</i>	x	OL672916
Fabaceae	Senna	<i>Senna surattensis</i>	x	OL672932
Fabaceae	Sesbania	<i>Sesbania grandiflora</i>	x	OL672949

Family	Genus	species	ITS2 sequence produced	Accession number
Malvaceae	Sida	<i>Sida cordifolia</i>	x	OL672933
Lamiaceae	Solenostemon	<i>Solenostemon sp</i>		
Lamiaceae	Solenostemon	<i>Solenostemon sp</i>	x	OL672943
Rubiaceae	Spermacoce	<i>Spermacoce hispida</i>		
Rubiaceae	Spermacoce	<i>Spermacoce sp</i>		
Asteraceae	Sphagneticola	<i>Sphagneticola trilobata</i>	x	OL672920
Verbenaceae	Stachytarpheta	<i>Stachytarpheta sp</i>		
Asteraceae	Synedrella	<i>Synedrella nodiflora</i>	x	OL672911
Malvaceae	Talipariti	<i>Talipariti tiliaceum</i>	x	OL672899 - OL672918
Malvaceae	Thespesia	<i>Thespesia populnea</i>	x	OL672921
Asteraceae	Titonia	<i>Tithonia diversifolia</i>		
Cucurbitaceae	Trichosanthes	<i>Trichosanthes sp 1</i>	x	OL672936
Cucurbitaceae	Trichosanthes	<i>Trichosanthes sp 2</i>	x	OL672942

Family	Genus	species	ITS2 sequence produced	Accession number
Asteraceae	Tridax	<i>Tridax procumbens</i>		
Turneraceae	Turnera	<i>Turnera ulmifolia</i>	x	OL672900
Fabaceae	Vigna	<i>Vigna sp</i>	x	OL672947
Asteraceae	Wollastonia	<i>Wollastonia biflora</i>	x	OL672912
Amaryllidaceae	Zephyranthes	<i>Zephyranthes sp</i>	x	OL672928

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In this work the third intermediate aim of the thesis has been achieved investigating the effects land use composition, configuration and of microclimatic condition related to urbanization on pollinator abundances, on the diversity of pollen they transport, and on the sugar content of nectar from spontaneous flowers. This investigation has been performed in the metropolitan city of Milan, one of the most urbanized area in Italy. My contribution is mainly related to the field sampling and the laboratory activities of pollen DNA metabarcoding.

Type of Article: Research article

CITY CLIMATE AND LANDSCAPE STRUCTURE SHAPE POLLINATORS, NECTAR AND TRANSPORTED POLLEN ALONG A GRADIENT OF URBANIZATION

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ABSTRACT

1. Urbanization gradients influence both landscape and climate and provide opportunity for understanding how species, especially plants and pollinators, respond to artificially driven environmental transitions.
2. Here, we investigated several aspects of pollination along an urbanization gradient in landscape and climate. We quantified wild hoverfly and bee abundances with trapping, standing crop of nectar with spectrophotometer, and the pollen transported by flower-visitors with DNA-metabarcoding, in 40 independent sites from seminatural to built-up areas in Northern Italy.
3. Linear and nonlinear relationships were detected along the urbanization gradient. Pollinator abundances increased until 22% of impervious surface, dropping by 34% after that, and it also decreased with green patch distance and urban park size. Thus, suburban landscapes host more pollinators than highly cemented or seminatural areas. Moreover, pollinators diminished by up to 45% in areas with low temperature seasonality: urbanized areas likely posing thermic stress. Furthermore, the sugar mass available in nectar increased by 91% with impervious cover, indicating that city nectars were less consumed or flowers more productive. Furthermore, the species richness of pollen decreased by 32% in highly urbanized areas, and with a high incidence of exotic plants, hinting for anthropized plant communities.
4. Synthesis and applications. Urbanization influences pollinator abundances, nectar resources and transported pollen. Pollinators are negatively affected by a thermally harsh climate in highly urbanized areas with isolated green areas and large parks. Suburban landscapes demonstrated the highest pollinator presence. In the city core, flowers contained more nectary sugar, while pollinators collected pollen from a small number of plants, mainly exotic. These findings highlight the influence of urban landscape structure and climate on pollinators and plants, showing that cities are heterogenous realities. Patterns from this study will serve as basis for pollinator-friendly planning and management of urban landscapes.

INTRODUCTION

Given the environmental change caused by cemented and built-up surfaces in contrast to adjacent ecosystems, urban areas are often considered as a separate macrohabitat for animal and plant communities (Bolger et al. 2000; Faeth and Kane 1978). Cities provide an artificial environment that creates an extreme transition (Lemoine-Rodríguez et al. 2020), causing several types of responses on biodiversity. These impacts affect a number of ecosystem processes (McIntyre et al. 2001), mainly by influencing changes in species interactions (Cohen et al. 2020; Geslin et al. 2013). As plants and pollinators play key roles in many ecosystem processes (Patel et al. 2020; Potts et al. 2016), gaining an understanding of how urbanization gradients shape aspects of pollination and pollinator ecology is of utmost importance.

Urbanization can be described as a gradient, with different consequences for plants and pollinators. For instance, plant diversity is higher at moderate urbanization (McKinney 2008), and suburban areas host higher wild bee and butterfly diversity than the city core (Banaszak-Cibicka and Żmihorski 2020; Kurylo et al. 2020). Surprisingly, the potential beneficial aspects of suburban landscapes on pollinator and plant communities have rarely been pointed out to-date (Wenzel et al. 2020; Harrison and Winfree 2015). Moreover, different pollinator types and life-history traits respond differently to urbanization (Wenzel et al. 2020) in spite of a generally high variation among studies (Buchholz and Egerer 2020). For instance, less Diptera Syrphidae than Hymenoptera are expected to occur in cities (Persson et al. 2020). Within Hymenoptera, built environments may change the composition of bee assemblages, with a high occurrence of solitary and above-ground nesting bees (Wilson and Jamieson 2019), while filtering big species out (Buchholz and Egerer 2020). Furthermore, fragment isolation may play a role, as a study found poorer pollinator assemblages in more isolated urban green areas (Tonietto et al. 2011). All these aspects highlight the need to further explore how pollinators respond to structural differences in urbanized landscapes.

Cities transform not only landscapes, but they also directly impact local climates (Kuttler 2008), thus likely triggering species physiological responses, even in plants and pollinators. Urban climate is usually warmer, it holds lower relative humidity and has higher precipitation than the surroundings (Kuttler 2008). The urban heat

island effect determines both high temperatures and lower temperature seasonality, a phenomenon that lasts both daily and across seasons (Marando et al. 2019). For instance, warmer cities impact plant physiology and phenology, triggering earlier flowering (Fisogni et al. 2020; Neil and Wu 2006). Also pollinator physiology can be affected (Hamblin et al. 2018). Intriguingly, a recent study showed that bees inhabiting city cores have higher thermic stress (Burdine and McCluney 2019). It is likely that the climatic impact posed by cities on plant and pollinator physiology are directly connected to patterns of diversity and abundances of those assemblages (Chown and Duffy 2015; Diamond et al. 2015). This is exemplified by studies showing that climatic features could affect some families of bees even more than landscape alteration (Kammerer et al. 2021). Therefore, it seems relevant to describe the responses of plants and pollinators not only as a function of city landscape alone but also by urban climatic variation.

In this study, we evaluated the effects of urbanization on several aspects of pollination and pollinator ecology, which are also relevant for ecosystem functioning (Biella, Akter, et al. 2019; Patel et al. 2020). We surveyed along a gradient of increasing urbanization in Northern Italy, a region characterized by a high proportion of built-up surfaces and remarkable climatic shift due to urban areas (Perini and Magliocco 2014). In order to address a mechanistic understanding of how an artificial gradient shapes pollination ecology and to connect local surveys to the structure of the surrounding area, we characterized landscape composition, its configuration and the climate.

We tested factors that were specifically selected based on hypothesized direct effects. Firstly, we measured pollinator abundance, as an indicator of habitat suitability (Bartholomé et al. 2020) and hypothesized that, along the urbanization gradient, pollinators may be dependent on the distribution and accessibility of suitable areas measured here as proportion of impervious cover and isolation of green spaces used for foraging (Steffan-Dewenter and Tscharrntke 1999). Within the context of the highly urbanized area, we also tested the role of city park size in order to further highlight the relationship with patch size as larger areas might serve as refugia for larger populations (Baldock et al. 2019). In addition, temperature variation along the urbanization gradient could impact flower visiting organisms and also determine their local abundance (Burdine and McCluney 2019; Hamblin et al. 2018) by affecting pollinator physiology (Colinet et

al. 2015). Secondly, we characterized the availability of nectar sugar mass, because nectar constitutes one of the main resources collected by flower visitors (Hicks et al. 2016). The reward quantity along the gradient would mainly be due to the secretion rate and thus to plant physiology, and its amount is partly determined by pollinator foraging rate (Corbet 2003). We hypothesize that the available nectar quantity could depend on the size of green areas because bigger patches may host richer communities (Collins et al. 2009; Dauber et al. 2010). In addition, plant productivity is often driven by precipitation and by the length of the thermally suitable season for growing, climatic parameters that vary with urbanization and that could impact nectar production (Mueller et al. 2020; Zipper et al. 2016). Thirdly, we considered the pollen diversity carried by flower-visitors, that were used here as a passive surveying of plant richness from the pollinator perspective (Biella, Tommasi, et al. 2019). In other words, we quantified the number of plant species visited by pollinators by studying the transported pollen, that is an important component of the total pollination rate (Bosch et al. 2009). Moreover, the advantage of using pollinators as passive samplers lies on the difficulty of sampling the vegetation of complex urban structures (e.g. tall buildings, balconies and private gardens) that impede traditional survey techniques. Here we described the transported pollen richness along the urbanization gradient (hence in relation to the impervious cover) and we also expected an influence by green-patch sizes, as it may determine local plant diversity (Collins et al. 2009; Dauber et al. 2010). We did not analyze climatic variables in this case as in anthropogenic areas pollen availability could be influenced by management (Aronson et al. 2017; Ibsen et al. 2020).

MATERIALS AND METHODS

Study area

Study sites were set in Northern Italy, mainly in the region surrounding Milan (Fig. 1) that is occupied by urbanized surfaces (about 38% of the area), intensive agricultural environments (ca. 53%), and natural forests, wet habitats and seminatural hay meadows (ca. 9%) (Regione Lombardia and ERSAF 2010). Study site locations were selected randomly with a GIS software (QGIS 3.6.2) and were distributed in an area of 1575 km² over a surface including the entire region of Milan and the urban parks of Milan city. We applied a minimum of 1 km distance between points to assure the independency of sampling sites (Phillips et al. 2019),

later confirmed by an autocorrelation analysis resulting not significant when testing correlation of putative predictors with themselves with Moran's I (P value > 0.05). We adjusted the exact sampling locations so that each would be located in either a urban park surrounded by "impervious" surfaces (i.e., concrete, asphalt, buildings) or at the margins of agricultural fields with a varying quantity of surrounding impervious surface or in seminatural hay meadows near forests (<1 km) with little amount of urbanization nearby (Fig. S1). Overall, 40 sites were surveyed, across the entire urbanization gradient (Fig. 1, Fig. S1).

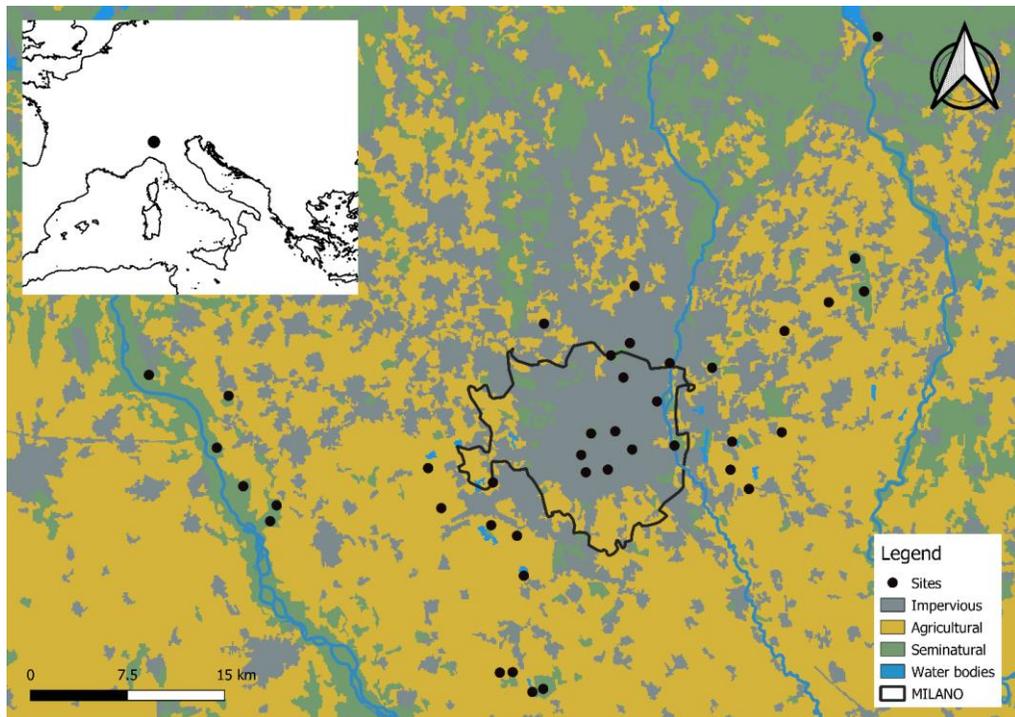


Figure 1 – Sampling sites. The distribution of sampling sites along a gradient of increasing urbanization in Northern Italy. The map also shows the Municipality of Milan, three major rivers and the main land uses of this region.

Landscape and local climate characterization

To characterize landscape composition, we measured the cover percentage of impervious (concrete-dominated) surfaces, urban green areas and seminatural lands (forests and hay meadows). To describe landscape configuration we focused on green and seminatural patches together and calculated Edge density (ED), the Euclidean nearest neighbor distance (ENN), and also the size of urban parks. ED

measures the total length of all edge segments per area unit and describes the fragmentation in size of the patches (i.e., highly fragmented when the perimeter is high relative to their area), while ENN describes the mean distance between patches in the landscape.

Temperature and precipitation vary with urbanization (see Figs S2-S3), we used 3-months averages of hourly temperature at 2-m and hourly precipitation sum between February and July 2019. We calculated an index of temperature seasonality as the coefficient of variation between spring and summer. Air temperature data were also used to calculate the Growing Season Suitability index (GSS), quantifying the length of the vegetative season that, together with precipitation, could impact the productivity of plant resources. All used variables were extracted from circular buffers of 1 km around the sampling sites, further methodological details are available in Appendix A1.

Pollinator abundance

Sampling of pollinator abundances took place during the peak flowering period for the study area, from mid-May to mid-June 2019, in 36 of the original 40 sites. Passive sampling took place for 24 hours at each site, with pan and sticky traps, that are complementary methods for sampling the same target insect groups. The pan trap consisted of a wooden stick holding three pan traps of yellow, blue and white bowls with UV reflection, containing water with 1-2 mL of soap to reduce surface tension, and the bowls height was adjusted specifically depending on grass tallness, so that the trap would be slightly higher than the height of the grass (Popic et al. 2013). Sticky traps were 15 * 20 cm yellow plastic board placed on poles at 1 m height on which pollinating insects remain glued (Sutherland et al. 2001). In each site, three sets of pan-traps were placed 10 m apart from each other; and five yellow sticky traps were placed at 5 m from the pan-traps. In this study, we counted the total abundance of bees and hoverflies (Hymenoptera: Anthophila and Diptera: Syrphidae) following (Bates et al. 2011). We did not count honeybees since their numerosity is only due to beehives in the vicinities. In the statistical analyses that followed we kept wild bees and hoverflies together and evaluated their total abundance as they belong to the same guild of pollinators (see Fig. S4).

Nectar sugar quantification

Standing crop of sugar mass in the nectar was measured from mid-May to mid-June in 35 sites. Flowers of two to three most abundant herbaceous flowering species were selected after an inspection of plant relative cover based on the number of stalks. We chose abundant plants as they should offer most of the local resources. Standing crop of nectar sugar mass is a measure of nectar quality available at a given time and it is a function of both plant secretion and of pollinator visitation frequency (Corbet 2003). As in Biella, Akter, et al. (2019), twenty to thirty blooming flowers for each species were taken from individual plants and the internal part of the corolla was washed in distilled water with a 100 μ l Hamilton syringe, the number of processed flowers was noted, samples were weighed and then frozen. Sugar quantification was performed using a spectrophotometer Cary 60 (Agilent Technologies, USA) and with Sucrose, D-Fructose and D-Glucose kits (Megazyme, Ireland). We used the sugar mass per flower for each given species in subsequent statistical analyses, which was calculated by dividing the sugar mass by the number of washed flowers of a species processed at a site (Biella, Akter, et al. 2019).

Pollen richness with DNA metabarcoding

In each sampled site, insects foraging on flowers were actively sampled for one hour by hand-netting and then stored in sterile Eppendorf tubes filled with ethanol 70%. In the laboratory, we randomly chosen a subset of the sampled sites (N=25) and analyzed the pollen from insect bodies with DNA metabarcoding following the protocol of Biella, Tommasi, et al. (2019). Full details of the laboratory protocol and bioinformatic processing are reported in Appendix A2. For each site, the number of plant species found in the pollen of all flower visitors was used as indication of pollen richness.

Statistical analyses

Putative predictors were chosen based on the ecological hypotheses outlined above. We used a repeated K-fold cross-validation for choosing if to fit linear or non-linear models by selecting the lowest RMSE (Root Mean Squared Error) between a linear GLM (Generalized Linear Model) and a non-linear GAM (Generalized Additive Model) associated to each predictor, ten repetitions of K=10 were performed and the mean RMSE was used for the evaluation (see Table S1).

To exclude collinear variables from the same analyses, we calculated the VIF index from preliminary regression models (Variance Inflation Factor, with an exclusion threshold of 4, Table S2) with the R package *car* (Fox and Weisber 2019). In the regression models listed below, predictors were square rooted to correct variable skewness and they were scaled to avoid different numerical ranges. Statistical significance was tested with likelihood-ratio tests. We analyzed the effects of landscape composition and configuration, and the summer-to-spring temperature seasonality on pollinator abundances in a Generalized Additive Model with the proportion of impervious land fitted with a smooth term, and the ENN and temperature variation as linear terms. These predictors were chosen because impervious cover describes the urbanization, the ENN indicates distance between patches used by pollinators, and temperature seasonality indicates the potential for thermic stress. This regression was fitted with Structural Equation Models (SEM), including the correlated errors between all predictors (Table S2), with the *piecewiseSEM* package with R (Lefcheck 2016). In addition, we modelled the relationship between pollinator abundances and urban park size with a GLM with data from urban parks (this predictor was log-transformed). In all cases, a Poisson family was used for error distribution with a log link function.

For sugars mass, we analyzed the effects of landscape variables and the contribution of urban climate. In particular, we fitted the sugar mass per flower as response and the proportion of impervious land, ED, the mean summer precipitation and the GSS as predictors in a Generalized Linear Mixed Model (GLMM), with a Gamma error distribution, the logarithm as a link function, and plant species and site identities as random intercepts. While impervious cover characterize the urbanization, the other variables describe the impact on plant productivity as ED explains the role of patch size on the diversity of plant communities, and the mean summer precipitation and GSS measure the amount of natural watering and length of the favorable season. This regression was fitted with Structural Equation Models (SEM), including the correlated errors between all predictors (Table S2).

We also analyzed the effect of the urban landscape on the transported pollen richness. We used the total pollen richness for each site as response variable in a GLM with the proportion of impervious land and ED as predictors, a Poisson family for error distribution and a log link function. These variables were used because impervious cover indicates the urbanization gradient and ED considers the role of

patch size in hosting plant diversity, while we did not expect a contribution from seasonal climate on the transported pollen. In addition, we investigated the connection between the geographical origin of plants and the landscape on the plant species of pollen transported. To do so, we used the Fourth Corner analysis to evaluate the relationship between plant incidence measured as the proportion of samples where a plant was found at a given site, plant traits as native of the sampled region, agricultural crop or exotic (Galasso et al. 2018), and site attributes as the proportion of impervious, seminatural and urban green cover. The analysis was performed with *ade4* package in R (Thioulouse et al. 2018) by setting 999 permutations of sites and species values for testing significances.

RESULTS

Pollinator abundances were nonlinearly dependent on the landscape variable of impervious cover, while it decreased linearly with green patches ENN (Table 1, Fig. 2). Pollinator abundance was positively linked to impervious cover until a threshold value of 22% cover after which the relationship became negative, decreasing by 34% (Fig. 2A). Increasing green patches ENN were associated with a decline in pollinator abundance of 27% across the range of the variable (Fig. 2B). Abundances of pollinators positively responded in a linear way to the seasonal variation in mean temperature between spring and summer (Fig. 2C), with the highest thermic seasonality in sites with medium to low impervious cover (see Fig. S2). Specifically, pollinators increased by 45% across the range of temperature variation. In city parks, the relationship between pollinator abundances and park size was negative (Table 1, Fig. S5), with a decline of 60% over the range of the studied urban parks (0.02 to 0.68 km²).

Sugar mass per flower was linearly dependent on impervious cover and on precipitations (Table 1, Figs 3A-B). Specifically, sugar mass increased by 91% across the range of impervious cover. However, it was not significantly dependent on green areas ED, or GSS or precipitation.

Table 1 – Estimated relationship between pollinator abundances, nectar sugar mass available and pollen species richness carried by pollinators and landscape and climatic variables. Significances are obtained with likelihood-ratio tests. SEM stands for Structural Equation Model; ENN indicates the Euclidean Nearest Neighbor distance of the green and seminatural areas; ED is the Edge Density, total length of all edge segments per unit of area; Growing Season Suitability index is indicated with GSS and it is the fraction of days between February and April with mean temperature above 10°C.

Response	Model type	Predictor	Regression slope (B _i)	Degree of freedoms; Chi squared χ^2	Significance P
Pollinator Abundances	Urbanization landscape and climate model (SEM)	Impervious land	Smoothed, see Fig.2	3.67; 57.30	< 0.001
		ENN	- 0.11	1; 16.25	< 0.001
		Temperature seasonality	0.29	1; 58.36	< 0.001
Pollinator Abundances	Urban parks (GLM)	Recreational Park size	- 0.532	1; 81.467	< 0.001
Nectar sugar mass	Urbanization landscape and climate model (SEM)	Impervious land	0.89	1; 6.72	< 0.01
		ED	-0.37	1; 2.29	0.13
		Summer precipitation	0.18	1; 0.92	0.33
		GSS	-0.25	1; 1.62	0.20
Pollen richness	Urbanization landscape (GLM)	Impervious land	-0.23	1; 11.29	< 0.001

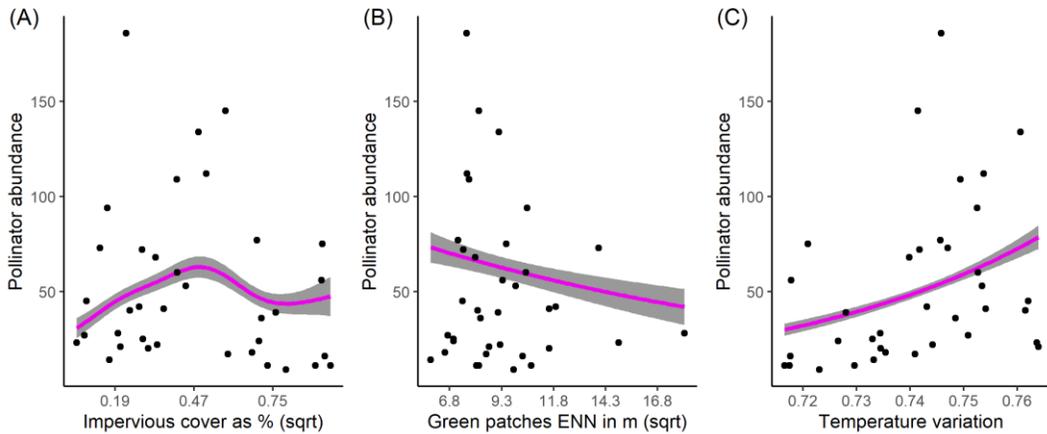


Figure 2 – Pollinator abundances along an urbanization gradient. Relationship of pollinator abundance with (A) impervious cover as a percentage of buffer area and (B) the “ENN”, mean distances of green patches; these predictors were square rooted (“sqrt”). (C) Relationship of pollinator abundances with temperature seasonality between spring and summer. Statistical outputs are reported in Table 1.

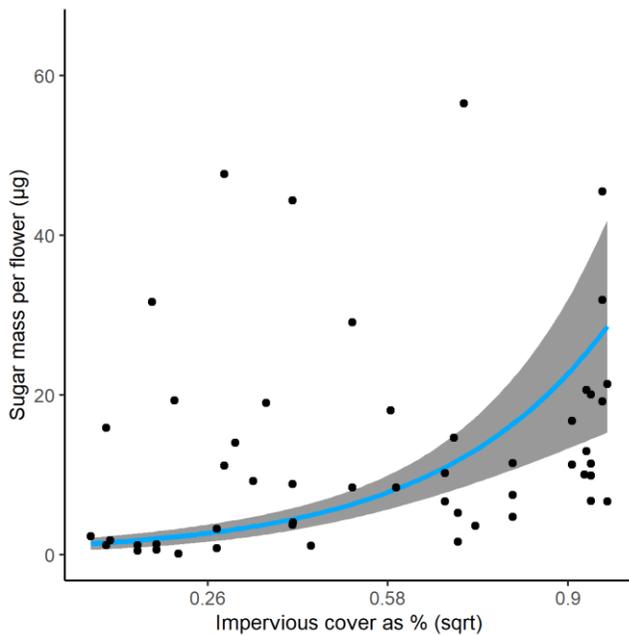


Figure 3 – Sugar mass in nectars along an urbanization gradient. The relationship of sugar mass (standing crop) per flower with impervious cover as a percentage of buffer area is shown. This predictor was square rooted (“sqrt”). The statistical outputs are in Table 1.

Table2 – The relationships between the type of plant species found in the pollen and landscape composition. Plants are indicated as “Native”, “Crops” or “Exotic”. Results of a Fourth corner analysis are shown, based on the frequency of plant presence in pollinator samples of each site.

Land use cover	Plant origin	Fourth corner statistic r	P value
Green areas	Exotic	0.203	0.025
Impervious	Exotic	0.139	0.205
Seminatural	Exotic	-0.194	<u>0.077</u>
Green areas	Native	-0.156	0.115
Impervious	Native	-0.076	0.499
Seminatural	Native	0.188	<u>0.091</u>
Green areas	Crop	-0.036	0.696
Impervious	Crop	-0.074	0.432
Seminatural	Crop	-0.025	0.77

The transported pollen richness showed a linear negative relationship with the impervious cover, and specifically by 32.5% across the range of the variable, and it was not significantly dependent on green areas ED (Table 1, Fig. 4). Most of the species in the pollen transported by pollinators were native to the region (66.1%). The incidence of exotic plants in the pollen samples was significantly higher at sites with higher cover of urban green areas (Table 2).

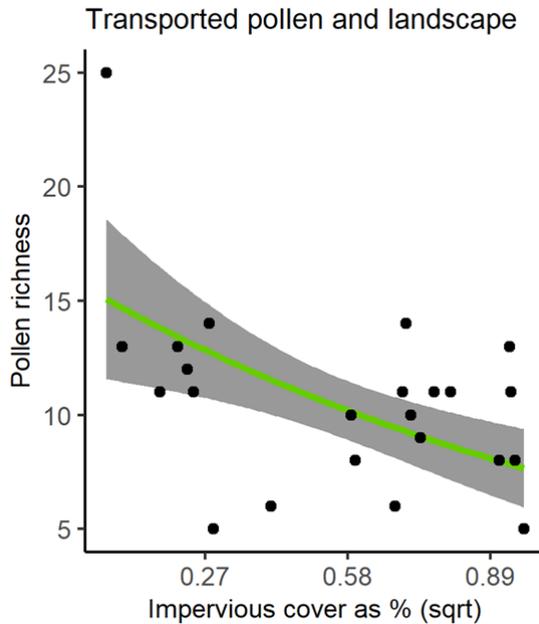


Figure 4 – Plant species richness in pollen from flower-visitor along an urbanization. The relationship between pollen richness for each site and impervious cover measured as surface percentage, in circular buffers of 1 km. This variable was square rooted (“sqrt”). The statistical outputs are indicated in Table 1.

DISCUSSION

Along the gradient of urbanization, landscape composition impacted pollinator abundance in non-linear ways. At low-to-medium levels of impervious cover, the pollinators were increasingly more abundant with urbanization, but landscapes with impervious surfaces higher than 22% in cover negatively impacted pollinator abundance. This threshold is comparable with another study from North America where butterfly abundance decreased when more than 25% of impervious cover occurred (Kurylo et al. 2020). Thus, cities are not homogenous entities, but some parts of the urban gradient may benefit pollinators (i.e., suburban areas) while others such as the heavily urbanized city cores do not, a pattern already observed in other studies (Banaszak-Cibicka and Żmihorski 2020; Buchholz and Egerer 2020). Interestingly, pollinators were lowly abundant even at very low impervious cover, perhaps either due to pollinator-unfriendly practices (e.g. agrochemicals, mechanical disturbance, low wildflower cover and diversity) or because pollinator

individuals are diluted over large open areas (Holzschuh et al. 2016). Moreover, the isolation of green patches negatively influenced pollinator abundances, in a linear way. This clearly indicates that the dispersion of green patches across a landscape may severely impact local pollinator abundances. This result recalls other studies showing that small and medium sized bees forage at close vicinities, for instance at maximal distance of 150 m (Hofmann et al. 2020; Zurbuchen et al. 2010). However, these studies were conducted in open landscapes, but in a urban setting it may be reasonable to expect lower home ranges given the presence of vertical obstacles (Harrison and Winfree 2015). Not only the type of landscape but also the size of city parks impacted pollinator abundances, as we recorded fewer pollinators with increasing park size. This result is comparable to what previously found in UK and in Germany (Baldock et al. 2019; Daniels et al. 2020). This may be due to low habitat quality (Bates et al. 2011) or a low population size diluted over a large area (Holzschuh et al. 2016). In spite of all these relationships, local conditions may play an important role in mitigating negative landscape impacts when nesting possibility and flowering resources are high (Delaney et al. 2020; Tommasi et al. 2021).

Temperatures also varied along the artificial gradient. Our analyses showed that pollinator abundances increased linearly with a rising temperature seasonality between spring and summer. This result indicates that pollinators are less abundant in sites where the climate is less variable between those seasons, and it contradicts previous ideas suggesting a link between a stable urban climate and pollinators (Baldock 2020). This is corroborated by a previous physiological study showing that wild bee species are affected by a high temperature where the impervious cover is high (Burdine and McCluney 2019). Furthermore, another study showed that bees avoid warmer areas in cities (Hamblin et al. 2018) and that warming may also reduce foraging activities (Kühnel and Blüthgen 2015). All together, these studies and our research indicate that pollinators may be sensitive to the harsh urban climate.

The impervious cover was positively associated with the standing crop of sugar mass available in the nectar of locally abundant plants, although independently to the fragmentation of the green areas (ED). These results showed a higher sugar mass available in urbanized areas than in non-urban sites. This result could be due to either higher secretion rate by plants or due to lower consumption by pollinators in cities (Corbet 2003). The latter possibility seems reasonable given a

lower pollinator abundance in the city core that may translate into a lower consumption rate. Conversely, if future actions will increase pollinator abundances even in the core of the city, the foragers will consume more nectar, and this will likely modify the observed trends with impervious surfaces. As it seems that a higher proportion of sugar mass available in cities is a prominent feature of a highly urbanized landscape hosting few pollinators, conservation plans aiming to increase pollinator abundance or richness would impact the nectar pattern observed. It may follow that, as cities provide higher sugar mass, they would sustain high pollinator abundances if the urban landscape was more pollinator friendly. Another relevant aspect to highlight is that nectar availability could vary with plant phenology. Thus, the nectar pattern we observed might be altered by different plants being in flower due to seasonal phenology (Hicks et al. 2016). Plant phenology might even cause seasonal gaps of nectar resources with severe implications for pollinators (Timberlake et al. 2019), and thus our results should be interpreted within the timeframe of our investigation.

In addition to the described patterns in pollinator abundance and nectar, a negative and linear relationship was detected between the impervious cover and the richness of plant species found in the pollen collected by pollinators. This means that the pollen from fewer plant species were transported by the pollinators during their foraging trips in highly urbanized sites. As pollen diversity on flower visitor bodies often reflects the local flowering plant diversity (Biella, Tommasi, et al. 2019; Bosch et al. 2009), this result reveals that urban parks of the study area are currently not offering to pollinators as diverse plant resources as areas outside the city. This result could be connected to the low plant diversity usually found in highly urbanized areas (McKinney 2008; Wittig and Becker 2010). Interestingly, we detected a higher incidence of non-native pollen in sites with a higher cover of urban green areas. This indicates that the urbanization deeply shaped the foraging patterns of pollinators, which more frequently visited exotic flowers. Concerningly, pollinators of urban areas carrying less diverse pollen richness, dominated by non-native species, may even have direct implications for plant reproduction (Cohen et al. 2020).

In this study, the urbanization gradient set important scenarios for understanding how plant and pollinators respond to habitat alteration and environmental transitions of urban landscape and climatic features. We detected that the artificial gradient shaped pollinator abundances, pollen species richness

transported by flower-visitors and sugar mass available in nectar in linear and nonlinear ways. These factors could have effects on plant reproduction, and on pollinator survival and nutrition. Importantly this study clarifies that suburban areas, generally characterized by cemented surfaces at medium-low density and green patches of low isolation, host a high pollinator abundance. It is important to note that pollinator abundances are often correlated to species richness (Vereecken et al. 2021), and thus it could be expected to find similar patterns when considering also pollinator richness. However, highly urbanized areas provide nectars richer in available sugars, while the pollen transported was less rich of plant species and frequently with non-native plants, compared to less urbanized areas. As the gradient is human-driven, future actions could modify the responses observed in this study. In particular, managing green areas incorporating practices that are more pollinator-friendly will likely increase pollinator abundances and their activity (Turo and Gardiner 2019). Thus, increasing the suitability of existing and future urban landscapes for plants and pollinators is a priority, given the relevance played for ecosystem services and even for human health (Smith et al. 2015).

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Author contribution

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

Supporting information

Appendix A1 – Describing local landscape and climate

To characterize landscape composition, circular buffers of 1 km were used to clip the 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). We grouped similar land uses into broader types mostly based on the Level 1 classification, and calculated the cover percentage of impervious (concrete-dominated) surfaces, urban green areas which are often frequently mown and seminatural areas including forests and hay meadows. In detail: DUSAF land use level 3 and 4 type "122", "124", "133", "1111", "1112", "1121", "1221", "1222", "12111", "12112", "12121", "12122", "12123", "12125" are categorized as Impervious; "3222", "3223", "314", "2241", "2242", "411", "2311", "2312", "3113", "3221", "3241", "3242", "31111", "31121" are categorized as Seminatural; "1122", "1123", "1411", "1412", "1421", "1422", "1423", "11231", "12124", "12126" Categorized as Green areas (Full explanation of codes is available at https://www.cartografia.regione.lombardia.it/metadata/Dusaf/doc/legenda_DUSAF5.pdf)

To describe landscape configuration, we focused on green and seminatural patches together and calculated Edge density (ED) and the Euclidean nearest neighbor distance (ENN). Park area was measured drawing polygons delimiting the entire area of the sampled urban recreational parks in the City of Milan with Google Earth (v 7.3.3.7786 <https://www.google.com/earth/index.html>). ED and ENN were obtained from a map of 5-m resolution, and packages *raster* (Hijmans and van Etten 2020) and *landscapemetrics* (Hesselbarth *et al.* 2019) were used in R (R Core Team 2017).

Temperature and precipitation were obtained from the regional environment agency in raster format, these are spatial interpolations derived from physical measures at a resolution of 1.5 km (<https://www.dati.lombardia.it/Ambiente/Interpolazione-osservazioni-orarie-precipitazioni-/stg2-h3gj> and <https://www.dati.lombardia.it/Ambiente/Interpolazione-osservazioni-orarie-temperatura-a-2/52ei-u66t>), later downscaled to a finer resolution of 100 m with bilinear interpolation. Both hourly air temperature in °C at 2-m and hourly

precipitation in mm were extracted for each buffer, between February and July 2019, that correspond to the sampling activities. We used these data to calculate 3-monthly means (*i.e.*, February-April, May-July) and, in particular, the air temperature was used to calculate the coefficient of variation in mean temperature between spring and summer ($(T_{\text{summer}} - T_{\text{spring}}) / \text{mean}(T_{\text{summer-spring}})$), an index for describing temperature seasonality that is relevant as a measure of the potential of thermic stress. Air temperature was also used to calculate Growing Season Suitability index (GSS), based on the fraction of days with mean temperature above 10°C during February-April (Zipper *et al.* 2016).

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Appendix A2– Protocol used for DNA metabarcoding and bioinformatic processing of pollen samples

Pollen was removed from insect bodies with a vortex shaker and, after taking out the insect, tubes were centrifugated. After removing the ethanol (with gentle pipetting first and then by evaporation in a chemical hood), grains were grinded with a Tissue Lyser® II (Qiagen©, Hilden, Germany) prior to immediate freezing in liquid nitrogen. Under a laminar flow cabinet, pollen DNA was extracted with Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following manufacturer's protocol. We amplified the nuclear internal transcribed spacer 2 region (ITS2), a plant DNA barcode region with high taxonomical resolution (Chen *et al.*, 2010). Primer sequence and further sample preparation are extensively explained in

Biella, Tommasi, et al. (2019). 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). The bioinformatic pipeline consisted in sequences pairing and pre-processing using QIIME2 2019.4 (Bolyen et al., 2019). After trimming primers, a quality filter was applied with an expected error of 2.0, and sequences out of 200 and 550 bp range were removed. The ESVs were extracted using DADA2 algorithm (Callahan et al., 2016). Each of the obtained 4520 ESVs were assigned to a taxonomic entity with Basic Local Alignment Search Tool (Camacho et al., 2009) against the public NCBI Genbank, based on the highest identity score. Taxonomical assignments were validated based on the known plant distribution (from GBIF database) and from the field observations of the authors of this study. The molecular identification of pollen yielded a high proportion of reliable assignments to the species level (76% of the *taxa*) while the rest was assigned to genus level.

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Supporting figures



Figure S1 – The three main environments that were sampled. From left to right: margins of agricultural fields with varying impervious surfaces nearby, urban parks surrounded by built-up surfaces, seminatural hay meadows near forests (<1 km) with little amount of urbanization nearby.

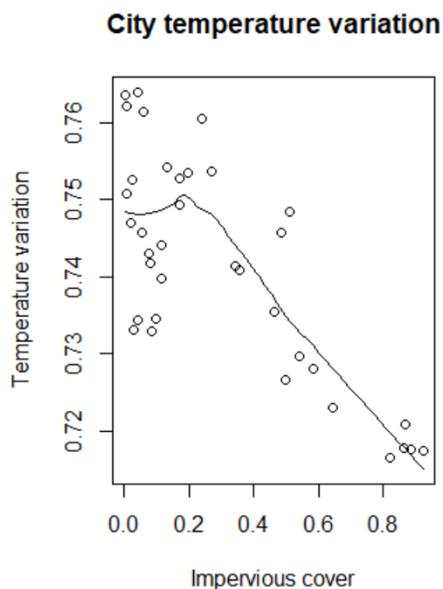


Figure S2 – Mean temperature variation between spring and summer and its relationship with urbanization measured as impervious cover. The line is a smoothed loess curve, the dots are the sampling sites.

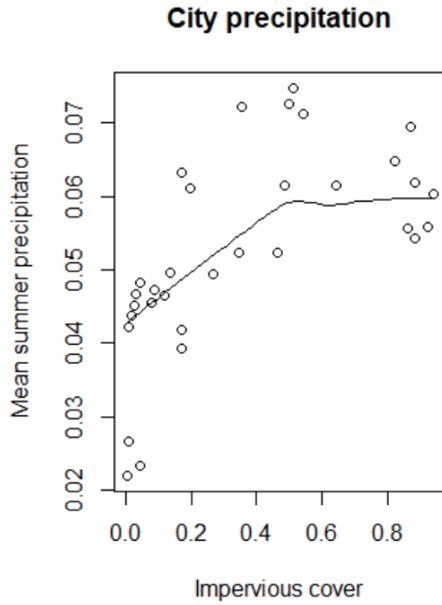


Figure S3 – Mean summer precipitation and its relationship with urbanization measured as impervious cover. The line is a smoothed loess curve, the dots are the sampling sites.

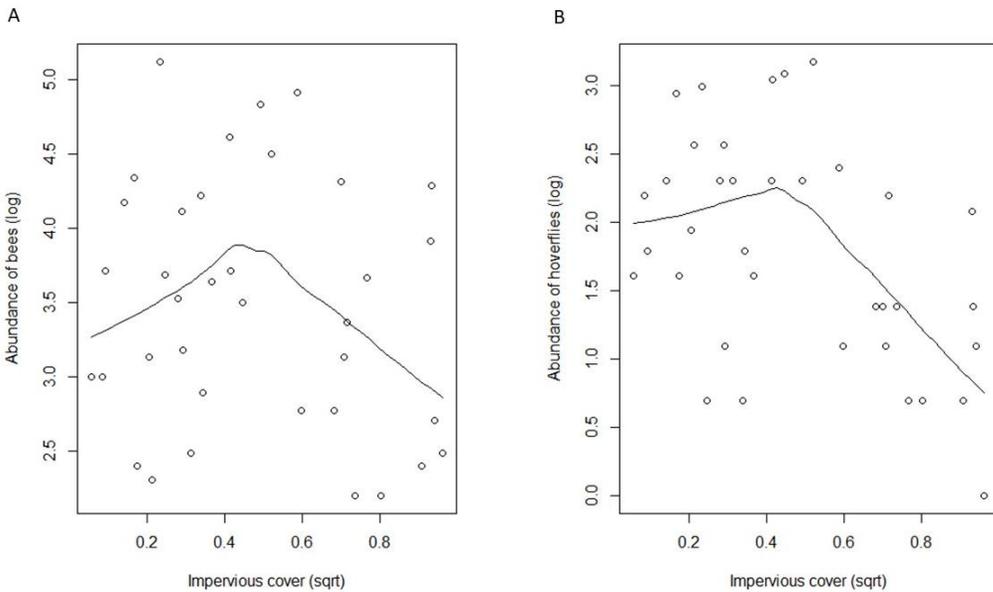


Figure S4 – Similar abundances patterns of (A) wild bees (excluding honeybees) and (B) hoverflies in response to urbanization measured as impervious cover. The line is a smoothed loess curve, the dots are data from the single sampling sites.

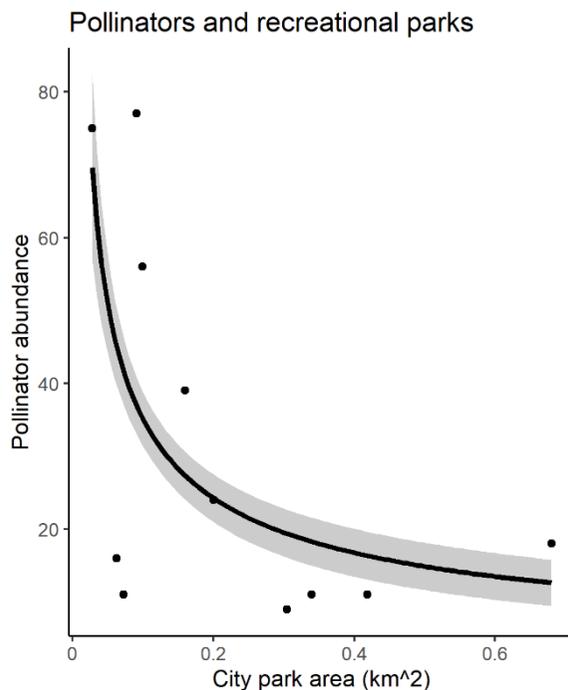


Figure S5 – Pollinator abundance in urban parks. Relationship of pollinator abundance with the area of parks of Milan city. Raw observations, and prediction with confidence interval from a GLM regression are shown.

Supporting tables

Table S1 – Repeated K-fold cross-validation for evaluating the best model between a linear a non-linear fit: GLM (Generalized Linear Model) and GAM (Generalized Additive Model), by choosing the one with lowest RMSE (Root Mean Squared Error); Ten repetitions of K=10 are performed for each model fitting each predictor singularly. ENN indicates the Euclidean nearest neighbor distance of the green and seminatural areas.

Response	Predictor(s)	RMSE _{GLM} - RMSE _{GAM}	Final model choice
Pollinator abundances	Impervious land	4.77	Non-linear
	ENN	-3.35	Linear
	Temperature seasonality	-4.59	Linear
Nectar sugar mass	Impervious land	-0.24	Linear
	ED	-0.79	Linear
	Summer precipitation	-0.14	Linear
	GSS	-0.17	Linear

Table S2 – Variance inflation factor (VIF) of predictors used in the Structural Equation Models (see methods), and Pearson correlation test resulted from the Structural Equation Models.

Response	VIF of Predictor(s)	Correlations
Pollinator Abundances	Temp. seasonality = 3.02; Impervious land = 3.81; ENN = 1.04;	Temp. seasonality and Impervious land: $r = -0.729$, $p < 0.001$; Temperature seasonality and ENN: $r = 0.04$, $p = 0.798$; Impervious land and ENN: $r = -0.19$, $p = 0.26$;
Nectar sugar mass	Precipitation = 2.11; Impervious land = 3.96; ED = 2.16; GSS = 2.27;	Precipitation vs Impervious land: $r = 0.68$, $p < 0.001$; Precipitation vs ED = 0.65, $p < 0.001$; Precipitation vs GSS = 0.45, $p < 0.01$; Impervious land vs ED = 0.67, $p < 0.001$; Impervious land vs GSS = 0.73, $p < 0.001$; ED vs GSS = 0.38, $p < 0.001$;

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8.4 Tommasi, N., Ferrari, A., Labra, M., Galimberti, A., & Biella, P. (2021). Harnessing the Power of Metabarcoding in the Ecological Interpretation of Plant-Pollinator DNA Data: Strategies and Consequences of Filtering Approaches. *Diversity*, 13(9), 437.

The following work has been conceived after the extensive use of the pollen DNA metabarcoding of the previous mentioned research, in the light of the growing number of studies employing the same approach. The first part of this work aimed at reviewing the use of pollen DNA metabarcoding, highlighting specific application and methodologies, but especially the approaches used to filter false positives and/or rare occurrence. In the second part of the work the consequences of different filtering strategies have been assessed by comparing changes in the observed biodiversity and interaction indices. I actively participated to the conceptualization of the work, data collection and analysis, as well as manuscript preparation and revision.

Type of Article: Review and Research article

HARNESSING THE POWER OF METABARCODING IN THE ECOLOGICAL INTERPRETATION OF PLANT-POLLINATOR DNA DATA: STRATEGIES AND CONSEQUENCES OF FILTERING APPROACHES

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Keywords: bioinformatics; cut-off thresholds; high throughput sequencing; rare taxa; false positives; molecular ecological network.

ABSTRACT

Although DNA metabarcoding of pollen mixtures has been increasingly used in the field of pollination biology, methodological and interpretation issues arise due to its high sensitivity. Filtering or maintaining false positives, contaminants, and rare taxa or molecular features could lead to different ecological results. Here, we reviewed how this choice has been addressed in 43 studies featuring pollen DNA metabarcoding, which highlighted a very high heterogeneity of filtering methods. We assessed how these strategies shaped pollen assemblage composition, species richness, and interaction networks. To do so, we compared four processing methods: unfiltering, filtering with a proportional 1% of sample reads, a fixed threshold of 100 reads, and the ROC approach (Receiver Operator Characteristic). The results indicated that filtering impacted species composition and reduced species richness, with ROC emerging as a conservative approach. Moreover, in contrast to unfiltered networks, filtering decreased network Connectance and Entropy, and it increased Modularity and Connectivity, indicating that using cut-off thresholds better describes interactions. Overall, unfiltering might compromise reliable ecological interpretations, unless a study targets rare species. We discuss the suitability of each filtering type, plead for justifying filtering strategies on biological or methodological bases and for developing shared approaches to make future studies more comparable.

INTRODUCTION

The study of Plant—Pollinator interactions is pivotal to address both theoretical and applicative issues, with important implications in evolutionary studies, conservation biology, and agrifood security, and it is relevant for providing reliable policies of land use management and mitigation of anthropogenic stressors [1,2,3,4].

Traditionally, studies of Plant—Pollinator interactions have been carried out with direct field observations of animal foraging activity during flower visitation [5,6]. However, to unveil Plant—Pollinator interactions, it is a valuable approach to classify the pollen grains carried on the pollinator's body [7,8]. This pollen might be accidentally picked and carried by flower visitors when they touch plant reproductive structures. Alternatively, it can be actively collected and accumulated in specialized structures such as the scopa or the corbiculae in the case of some bee species. The identification of the transported pollen allows discovering of the foraging “history” of flower visitors prior to a sampling event. In this way, it is possible to retrieve complete behavioural and ecological information on flower resource exploitation and to address ecological research questions in a potentially fine detail. To achieve pollen identification, classical palynology based on morphology has traditionally been used. This approach could provide lot of information about sample composition. However, it requires high expertise with pollen morphological assessment and, based on the operator's expertise, can be time-consuming [9,10]. In addition, a detailed taxonomic resolution through morphological criteria could be limited by the lack of diagnostic characters among taxa [11]. The morphological approach could be useful in cases of a low number of pollen samples to be identified or for gathering quantitative information on pollen amounts [12].

In the last decade, morphological difficulties have progressively been overcome by using DNA-based approaches that significantly reduce the time required for pollen identification [13,14]. Recent developments in DNA sequencing technologies, especially those based on High-Throughput Sequencing (HTS), made it possible to analyse the taxonomic composition of complex DNA matrices. For example, mixed pollen samples can be characterized by using standard DNA barcode regions in a so-called DNA metabarcoding approach [15,16]. For pollen-based studies, DNA metabarcoding is becoming a standard approach, being employed not only for the

characterization of the pollen retrieved from animal bodies (see e.g., [17]), but also in the analysis of other kinds of samples such as cavity nests [18], honey [19], sediments [20,21], and forensics [10,22]. In the context of Plant—Pollinator interactions, the type of data retrieved from pollen DNA metabarcoding could potentially shed light on the foraging habits of flower visitors or evaluate the complexity and resilience of the interaction networks. This methodological revolution not only improved ecological knowledge, but it also offered new insights into the development of effective conservation and restoration actions [9].

Given the astounding number of sequences (hereafter “reads”) [23,24], the data from HTS techniques require a proper bioinformatic pipeline. This is a critical phase of the dry lab activities and usually consists in (i) the assembly of paired-end reads resulting from bidirectional sequencing of the DNA templates, (ii) the analysis of the variation among sequences and the clustering of molecular features (e.g., Operational Taxonomic Units OTUs sensu [25] or Exact Sequence Variants ESVs sensu [26]), and (iii) the removal of chimeras, artifacts, and spurious sequences [27]. However, this bioinformatics process does not completely solve all the potential biases. Additional artifacts, hereafter referred to as false positives, result from clusters of molecular features (i.e., OTUs and ESVs) generated as a consequence of inaccuracies during field sampling operations (e.g., cross-contamination among samples), laboratory processing (e.g., contamination of DNA extraction or amplification reagents), or from some steps of the bioinformatics analysis (e.g., taxonomic misidentification of molecular features) [24,28]. The presence of infrequently detected molecular features or taxa might add further background noise in the output of a DNA metabarcoding pipeline. Given the extreme sensitivity of DNA metabarcoding, it is crucial to filter out false positives and contaminants, which could significantly alter the reconstruction of samples composition. Moreover, rare features or taxa should be treated consciously during the postsequencing bioinformatics processing and possibly removed, depending on the study aims and the required sensitivity of the analysis [27,28,29,30]. However, the resulting species composition of a sample could be biased by the disapplication or misapplication of cut-off thresholds. For instance, DNA metabarcoding could detect the occurrence of particularly infrequent taxa, which may be of interest in some specific cases (e.g., tracking the origin of a sample based on rare pollen). On the other hand, these may produce a great

impact on the ecological interpretation of results, especially when reads counts are converted into presence/absence data. Such a situation could lead to the overestimation of the generalist attitudes of the investigated pollinators and to misleading ecological interpretations.

The application of an appropriate cut-off threshold to filter the DNA metabarcoding data from the signal of possible false positives and rare taxa or features is therefore a critical step of the bioinformatics pipeline. Although some studies have not applied any cut-off threshold, different approaches for filtering false positives and rare taxa or features have been used so far in recent literature. In practice, some studies applied fixed cut-off thresholds, such as a defined number of reads used as reference level for accepting a molecular feature or taxon in a sample (e.g., [31]). Other studies employed proportional cut-off thresholds, where molecular features or taxon are discarded if represented by less than a certain percentage of the total reads of a sample (e.g., [32]). Alternatively, statistical approaches have been used for estimating a variable threshold based on Receiver Operator Characteristic (ROC) curves, thus depending on the distribution of reads among molecular features or taxa within a sample [17]. This highlights the absence of agreement on whether and how to prune a DNA metabarcoding output. However, to date, no studies have investigated the effect of each of the abovementioned filtering strategies on molecular datasets concerning pollen samples (or honey) and Plant—Pollinator interactions.

In this study, we investigated and summarized the criteria and the strategies adopted for filtering out the false positives and rare features or taxa, focusing on published studies on pollen DNA metabarcoding. Moreover, we aimed at evaluating the direct ecological effects of the most commonly applied filtering methods on publicly available datasets of pollen/honey DNA metabarcoding. To do this, we measured how unfiltering or different cut-off thresholds impacted (i) plant species composition and species richness and (ii) the interactions among plants and pollinators described by network indexes. With these aims, we evaluated how the different filtering strategies could alter the identification of species and of interactions, and thus the ecological interpretation of the results.

METHODS

Filtering Taxa from Pollen DNA Metabarcoding: Literature Overview

To revise the types of filtering and the methodology applied in the scientific literature used to remove (or not) false positives and rare taxa or features, bibliographical research was conducted in Scopus using the following keywords: “DNA” + “metabarcoding” + “pollen”. Within the results of the query, we selected only peer-reviewed original published articles that dealt with pollination, pollinator diet (pollen and honey), and plant—pollinator interactions by using a DNA metabarcoding approach (we excluded reviews, news, views, opinions, perspectives papers, and studies on airborne pollen or other pollen matrices when unrelated to pollinators). We selected studies spanning between 2012, when the term DNA metabarcoding was proposed for the first time [16], and 2021 (last update on 9 May 2021). The retrieved articles were used to create a summarizing table (Table 1) including: (i) the type of sample from which the DNA was extracted, (ii) the studied organism, (iii) the details of the filtering applied, and (iv) the DNA barcoding markers used to achieve the amplification reaction.

Source	Sample type	Organism	Type of Cut-Off Threshold	Detail on the Application of the Cut-Off Threshold	DNA Barcode Marker(s)	Dataset Used in This Study
Baksay et al. (2020) [33]	Mock pollen samples	-	Mixed	Sequences with a count of ≤ 10 , with no variants and with a count $< 5\%$ of the total reads per sample	ITS1, <i>trnL</i>	
Bänsch et al. (2020) [34]	Pollen from legs	<i>Apis mellifera</i> , <i>Bombus</i> spp. (Apidae)	Not specified	-	ITS2	

Source	Sample type	Organism	Type of Cut-Off Threshold	Detail on the Application of the Cut-Off Threshold	DNA Barcode Marker(s)	Dataset Used in This Study
Bell et al. (2017b) [35]	Mock pollen samples	-	Negative controls	Removed identifications occurring at a frequency lower than those obtained in negative controls (isolation negative control = 34 reads, PCR negative control = 30 reads)	ITS2, <i>rbcl</i>	
Bell et al. (2017a) [12]	Pollen from the whole body	Hymenoptera: Anthophila	Negative controls	Removed taxonomic classifications recorded from reads fewer than the maximum number of a negative control (21-936 <i>rbcl</i> and 42-1124 ITS2)	ITS2, <i>rbcl</i>	X
Bell et al. (2019) [24]	Mock pollen samples	-	Negative controls	Threshold based on the maximum sequence count from any negative control (11 and 34 ITS2, 8 and 30 <i>rbcl</i>)	ITS2, <i>rbcl</i>	X

Source	Sample type	Organism	Type of Cut-Off Threshold	Detail on the Application of the Cut-Off Threshold	DNA Barcode Marker(s)	Dataset Used in This Study
Beltramo et al. (2021) [36]	Honey	<i>Apis mellifera</i> (Apidae)	Proportional	Removed OTUs with <0.2% of the reads	trnL	
Biella et al. (2019) [17]	Pollen from legs	<i>Bombus terrestris</i> (Apidae)	Variable: statistical based	Receiver Operating Characteristics (ROC)	ITS2	X
Danner et al. (2017) [37]	Pollen from legs	<i>Apis mellifera</i> (Apidae)	Proportional	Removed species <1% of the relative reads count per sample	ITS2	
DeVere et al. (2017) [38]	Honey	<i>Apis mellifera</i> (Apidae)	Not specified	-	<i>rbCL</i>	X
Elliott et al. (2021) [39]	Pollen from legs or scopa	Hymenoptera: Apidae, Megachilidae, Colletidae	Proportional	Removed taxa <1% of all reads per plant taxon for each bee species	<i>rbCL</i>	
Fahimee et al. (2021) [40]	Pollen from the whole body	<i>Heterotrigona itama</i> (Apidae)	Fixed— Not proportional	Removed OTUs with <2 reads	trnL	
Galliot et al. (2017) [41]	Pollen from the whole body	Diptera, Hymenoptera, Coleoptera, Lepidoptera	Negative controls	Threshold of 3 reads based on negative controls	ITS2	
Gous et al. (2019) [42]	Pollen from the scopa	<i>Megachile venusta</i> (Megachilidae)	Proportional	Removed taxa <0.1% of total reads number per sample	ITS1, ITS2, <i>rbCL</i>	
Gous et al. (2021) [43]	Pollen from the scopa	<i>Megachile</i> spp. (Megachilidae)	Proportional	Removed taxa <0.1% of total reads number per	ITS2	

Source	Sample type	Organism	Type of Cut-Off Threshold	Detail on the Application of the Cut-Off Threshold	DNA Barcode Marker(s)	Dataset Used in This Study
				sample		
Hawkins et al. (2015) [44]	Honey	<i>Apis mellifera</i> (Apidae)	Fixed— Not proportio nal	Removed taxa <10 sequences	<i>rbCL</i>	
Jones et al. (2021) [45]	Honey	<i>Apis mellifera</i> (Apidae)	Fixed— Not proportio nal	Singletons discarded	ITS2, <i>rbCL</i>	X
Khansaritor eh et al. (2020) [46]	Honey	<i>Apis mellifera</i> (Apidae)	Not specified	-	ITS2, <i>rbCL</i>	
Leidenfrost et al. (2020) [47]	Pollen from legs	<i>Bombus terrestris</i> (Apidae)	Not specified	-	ITS2	
Lucas et al. (2018a) [48]	Pollen from the whole body	Syrphidae	Not specified	-	<i>rbCL</i>	
Lucas et al. (2018b) [49]	Pollen from the whole body	Syrphidae	Not specified	-	<i>rbCL</i>	
Lucek et al. (2019) [50]	Honey	<i>Apis mellifera</i> (Apidae)	Fixed— Not proportio nal	5 reads per sequences cluster	ITS2	X
Macgregor et al. (2019) [51]	Pollen from proboscid	Lepidoptera (moths)	Negative controls	Threshold of 50 reads based on positive and negative controls	<i>rbCL</i>	
Nürnberg er et al. (2019) [52]	Pollen from legs	<i>Apis mellifera</i> (Apidae)	Not specified	-	ITS2	

Source	Sample type	Organism	Type of Cut-Off Threshold	Detail on the Application of the Cut-Off Threshold	DNA Barcode Marker(s)	Dataset Used in This Study
Peel et al. (2019) [53]	Pollen from legs	<i>Apis mellifera</i> , <i>Bombus</i> spp. (Apidae)	Proportional	Removed taxa <1% of the total assigned long reads per sample	Genomic DNA	
Piko et al. (2021) [54]	Pollen from the whole body	<i>B. terrestris</i> , <i>B. pascuorum</i> , <i>B. lucorum</i> (Apidae)	Mixed	Removed taxa <100 reads each sample and <1% of the total read count per sample	ITS2	
Pornon et al. (2016) [55]	Mock pollen samples, Pollen from whole body	<i>Hippeastrum</i> sp., <i>Chrysanthemum</i> sp., <i>Lilium</i> sp.; Diptera, Hymenoptera, Coleoptera, Lepidoptera	Mixed	Removed taxa <0.1% of the most common sequences and <10 reads each sample	ITS1, <i>trnL</i>	
Pornon et al. (2017) [56]	Pollen from the whole body	Diptera, Hymenoptera, Coleoptera, Lepidoptera	Fixed— Not proportional	Sequences less than <1000	ITS1, <i>trnL</i>	
Pornon et al. (2019) [31]	Pollen from the whole body	Syrphidae, Empididae, Apidae	Fixed— Not proportional	Sequences less than <1000	ITS1, <i>trnL</i>	
Potter et al. (2019) [57]	Pollen from the whole body	Hymenoptera: Anthophila	Not specified	-	<i>rbcl</i>	
Richardson et al. (2015a) [19]	Pollen from legs	<i>Apis mellifera</i> (Apidae)	Not specified	-	ITS2	
Richardson et al. (2015b) [58]	Pollen from legs	<i>Apis mellifera</i> (Apidae)	Not specified	-	ITS2, <i>rbcl</i> , <i>matK</i>	

Source	Sample type	Organism	Type of Cut-Off Threshold	Detail on the Application of the Cut-Off Threshold	DNA Barcode Marker(s)	Dataset Used in This Study
Richardson et al. (2019) [59]	Pollen from legs	<i>Apis mellifera</i> (Apidae)	Proportional	Removed genera identified with only one marker and taxa with proportion of sequences <0.01%	ITS2, <i>rbcl</i> , <i>trnL</i> , <i>t rnH</i>	
Richardson et al. (2021) [60]	Pollen from legs	<i>Apis mellifera</i> (Apidae)	Proportional	Removed genera identified with only one marker and with <0.001 proportional abundance of sequences	ITS2, <i>rbcl</i> , <i>trnL</i>	
Sickel et al. (2015) [61]	Pollen from nest	<i>Osmia trunctorum</i> (Megachilidae)	<i>bicornis</i> , <i>O.</i>	Proportional	Removed taxa <0.1% of reads per sample	ITS2
Simanonok et al. (2021) [62]	Pollen from legs	<i>Bombus affinis</i> (Apidae)	Mixed	Removed OTU <10 reads and taxa with <2% reads per sample	ITS2	
Smart et al. (2017) [63]	Pollen from legs	<i>Apis mellifera</i> (Apidae)	Fixed—Not proportional	Removed taxa <50 reads	ITS1, ITS2	
Suchan et al. (2019) [64]	Pollen from the whole body	<i>Vanessa cardui</i> (Lepidoptera)	Fixed—Not proportional	Removed taxa <100 reads per sample	ITS2	

Source	Sample type	Organism	Type of Cut-Off Threshold	Detail on the Application of the Cut-Off Threshold	DNA Barcode Marker(s)	Dataset Used in This Study
Swenson et al. (2021) [65]	Mock pollen samples	-	Mixed	Removed taxa <0.1% of the sample reads of ITS1 and ITS2; removed taxa occurring at a lower frequency than those obtained from negative controls	ITS1, ITS2, <i>rbcl</i>	
Tanaka et al. (2020) [66]	Pollen from honeycomb	<i>Apis mellifera</i> (Apidae)	Not specified	-	<i>rbcl</i>	
Tommasi et al. (2021) [67]	Pollen from the whole body	Hymenoptera: Anthophila, Syrphidae	Diptera: Not specified	-	ITS2	X
Tremblay et al. (2019) [68]	Pollen from legs	<i>Apis mellifera</i> (Apidae)	Fixed—Not proportional	Removed taxa <100 reads	ITS2	
Vaudo et al. (2020) [69]	Pollen from nest	<i>Osmia cornifrons</i> (Megachilidae)	Proportional	Removed taxa <1% sample read abundance and genera <0.3% of the total read counts per site across all sites	ITS2	X

Source	Sample type	Organism	Type of Cut-Off Threshold	Detail on the Application of the Cut-Off Threshold	DNA Barcode Marker(s)	Dataset Used in This Study
Wilson et al. (2021) [32]	Pollen from nest	<i>Tetragonula carboniaria</i> (Apidae)	Proportional	Removed taxa identified in blank controls with abundance <1% of the relative read abundance in real sample	ITS2, <i>rbcL</i>	

Table 1. List of published studies subjected to review, including details on referencing, used samples in the DNA metabarcoding analysis, the organisms from which the pollen samples were collected, and the cut-off threshold with a brief explanation of the filtering actually applied. Additional information is given on the DNA barcode marker(s) used and on whether the dataset was included in the present study.

Evaluating the Consequences of Filtering (or Not) Taxa

To evaluate how the application (or not) of different cut-off thresholds could lead to changes in the results and their interpretation, we retrieved publicly available DNA metabarcoding datasets based on the ITS2 DNA barcode marker (that is the most used in pollen DNA metabarcoding studies) from the previously mentioned literature search. Only those datasets which were not preliminarily filtered were kept for our analysis (see Table 1). In detail, we retrieved published nonfiltered datasets (hereafter named as “no cut”, equivalent to a 0-reads threshold), and we derived several subsequent filtered versions by separately applying three different approaches for filtering false positives and rare taxa or features. The filtering approaches were chosen based on utilization frequency in the literature or on their biological reliability (i.e., the ROC approach). Specifically, the first method is based on a fixed threshold, and it removes from a sample the molecular features or taxa represented by less than 100 reads (hereafter “fixed 100 reads”), thus

mimicking studies where exclusion thresholds are based on reads found in sequencing blanks (e.g., [12]). The second method is proportional and discards what is represented in a sample by a number of reads lower than 1% of the total sample count of reads (hereafter “proportional 1%”) as used for example in [37]. The third one estimates a cut-off threshold accounting for the distribution of reads among molecular features, thus providing a customized proportion for each sample through the statistical ROC curve approach, as indicated in [17] (hereafter “statistical ROC”). This strategy is commonly applied in several disciplines, and it was specifically proposed for the detection of false positives [70]. We applied the ROC approach in the same way as it was done in the pollen-based literature, thus following the procedure of [17]; see Supplementary Material Text S1 for a script, although different implementations of ROC are possible and they might affect the final estimations. We associated a variable coded as “negative” or “positive” to each taxon of a sample. Specifically, “negative” was assigned if its reads were 0; otherwise, “positive”. We fitted a Generalized Linear Regression with an over dispersed Poisson distribution (quasi-Poisson) for each sample to model the distribution of the amount of reads per taxa (quantitative response) between “positives”/“negatives” (categorical predictor). Fitting a regression is a necessary step for later estimating the false positives of a sample that otherwise are usually not known in DNA metabarcoding data. The predicted reads distribution was processed with the pROC package [71] (in the R environment) that uses the roc function to build ROC curves between the reads per sample estimated by the GLM and true “positives”/“negatives” (those used to fit the regression). The optimal threshold of reads below which taxa should be excluded was obtained with the function coord in the same package based on the Youden’s J statistic [72] (see [71] for further details).

For each dataset, changes in plant species composition and species richness (that was standardized for the maximum number of species observed in a sample) for each pollen sample was evaluated in response to the type of filtering used (i.e., no cut, fixed 100 reads, proportional 1%, and statistical ROC). For the comparison of pollen species composition, we used a Permutational Manova based on distance matrices (with Jaccard distance index), which is an analysis of variance that uses a permutations test with pseudo-F ratio [73]. This analysis was performed through the adonis function with R-package vegan [74], where each dataset was analysed independently. The effect of the different cut-off thresholds on species richness

was evaluated through a Generalized Linear Mixed Model (GLMM) with species richness as response variable and the type of filtering used (i.e., no cut, fixed 100 reads, proportional 1%, and statistical ROC) as covariate. The identity of the pollinator animal nested within the dataset was set as a random effect.

Network indices describing the interactions between plants and pollinators were calculated. Specifically, the analysed indices were Connectance (i.e., proportion of possible links actually recorded), Modularity (i.e., a measure of how interactions are distributed into modules, where species within modules mostly interact with each other), and Shannon Entropy (i.e., a measure of the overall diversity and complexity in the interactions of a network). Furthermore, at the level of a single individual pollinator, the Connectivity index was calculated. This index of centrality quantifies the putative central role of an individual or of a species in connecting different parts of the whole network [75]. It could provide information in ranking individuals or species according to their contribution to the stability of the interactions and the cohesion among network participants. Network indices were calculated through the R-package bipartite (specifically for Connectance, Modularity, and Entropy) and rnetcarto (for Connectivity) [76,77]. For this purpose, only those datasets originated from direct characterization of pollinator foraging were used, excluding a study on mock samples [24] and a study with an incomparable experimental design [38] that instead were used in the other analyses. Changes in interaction indices at the network level (Connectance, Modularity, Entropy) were evaluated through either a Linear or a Generalized Mixed Model depending on the distribution and range of the response variable. The type of filtering used was included in the models as covariate, and the dataset identity, as random effect. The individual level (i.e., samples) Connectivity was analysed as response variable, the type of filtering used, as covariate in interaction with the normalized degree of the pollinator individuals. This normalized degree was calculated as the number of plant species found in each sample divided by the overall number of plants. The inclusion of the normalized degree in this analysis allowed us to describe the variation of Connectivity across the entire specialism–generalism spectrum of an individual and in relation to the applied filtering approach. In this case, the sample identity nested within the dataset was included in the model as a random effect.

For all the mentioned regression analyses, a comparison among the adopted strategies of filtering was performed through a post hoc test (Tukey's HSD test).

All the statistical analyses explained above were carried out with R (Version 3.6.1 of R).

RESULTS

Filtering Taxa from Pollen DNA Metabarcoding: Literature Overview

Overall, 43 research articles on pollen DNA metabarcoding were found and reviewed concerning the strategy of filtering of false positive and rare taxa or features (Table 1). About one quarter of studies did not apply any filtering approach, while the remaining ones applied at least a filtering type. Specifically, the proportional cut-off threshold was the most applied method (11 studies, 28%). In these studies, the cut-off threshold calculated as 1% of the number of reads produced by each sample was the most recurrent. Other filtering types were less common. Only one study used a statistical approach (i.e., the ROC curve; [16]) to set a proportional cut-off threshold. Nine studies (21%) used a fixed number of reads chosen arbitrarily as cut-off threshold (e.g., 100 or 1000 reads), while five other studies (12%) used the number of reads produced by negative controls to set the threshold to remove false positives and rare taxa or features. Finally, five studies (12%) used more than one filtering approach simultaneously. Details and a brief explanation of the strategies applied to set the cut-off threshold for the reviewed studies are reported in Table 1.

Twenty-eight published studies (65%) recovered the pollen samples from the whole insect's body or from specific body parts such as scopa and corbiculae. Four studies (9%) focused on pollen stored in cavity nests or in hives, while five (12%) investigated mixed pollen mock samples to address methodological issues (e.g., the optimization of DNA extraction or quantitative use of DNA metabarcoding reads). Finally, six studies (14%) analysed the taxonomic composition of honey by looking at the pollen grains contained in it.

Most of these studies (65%) used the ITS2 marker as a DNA barcode region for species identification, although in some these cases (28%), this marker was combined with other barcode loci (e.g., rbcL).

Evaluating the Consequences of Filtering (or Not) Taxa

From the 43 reviewed studies, eight nonfiltered and publicly available ITS2 DNA metabarcoding datasets were retrieved. Among these, four were obtained by processing the pollen found in nests or carried on insects' bodies [17,35,67,69].

Three datasets contained data from honey samples [38,45,50], and one was obtained from the analysis of pollen mock samples [24].

Significant changes in the composition of pollen samples depending on the filtering approach are summarized in Table 2. Specifically, the main differences occurred between the no-cut and all the other filtering approaches, in all datasets (Table 2). Minor changes in community composition among fixed 100 reads, proportional 1%, and statistical ROC were only occasionally found (Table 2).

Plant species richness inferred from pollen samples was significantly influenced by the filtering approach ($X^2 = 468.22$, $p < 0.001$). Specifically, higher species richness per sample was found in the unfiltered type (i.e., no cut) compared to all the other filtering approaches. A significant difference between the proportional 1% and the statistical ROC approaches was also found, with the latter reducing species richness even more (Figure 1a, Table 3).

Dataset	F-Value	Significance p of Full Model	Significance p of Pairwise Comparisons					
			Proportional 1% vs. No Cut	Fixed 100 Reads vs. No Cut	Proportional 1% vs. Fixed 100 Reads	Statistical ROC vs. No Cut	Statistical ROC vs. 1%	Statistical ROC vs. Fixed 100 Reads
Tommasi et al. (2021) [67]	0.819	0.806	1	1	1	0.031	0.314	0.045
Bell et al. (2017a) [12]	87.264	0.001	0.001	0.001	0.14	0.001	0.001	0.001
Bell et al. (2019) [24]	39.817	0.001	0.001	0.001	0.01	0.001	0.658	0.001
Biella et al. (2019) [17]	29.671	0.001	0.001	0.001	0.035	0.001	0.725	0.008
Jones et al. (2021) [45]	6.538	0.001	0.001	0.001	0.944	0.001	0.233	0.855
Lucek et al. (2019) [50]	5.465	0.001	0.001	0.001	0.038	0.001	0.975	0.058
DeVere et al. (2017) [38]	2.415	0.024	0.004	0.212	0.538	0.003	0.704	0.152
Vaudo et al. (2020) [69]	11.553	0.001	0.001	0.001	0.556	0.001	1	0.578

Table 2. Comparison of cut-off thresholds applied on pollen species composition of samples from several datasets, based on Permutational Manova. Dataset names (entitled with main author and year; see Table 1 for further details) are reported in the first column “Dataset”. The column “F-value” reports the pseudo-F ratio value and the associated significance p ($\alpha = 0.05$). Significant cases are reported in bold.

Variable	Comparison	Estimated Difference	Significance <i>p</i>
Species richness	No cut—Fixed 100 reads	0.383	<0.001
	Proportional 1%—Fixed 100 reads	0.043	0.178
	Statistical ROC—Fixed 100 reads	-0.013	0.934
	Proportional 1%—No cut	-0.340	<0.001
	Statistical ROC—No cut	-0.396	<0.001
	Statistical ROC—Proportional 1%	-0.055	0.040
Connectance	No cut—Fixed 100 reads	0.660	0.008
	Proportional 1%—Fixed 100 reads	-0.068	0.991
	Statistical ROC—Fixed 100 reads	-0.131	0.941
	Proportional 1%—No cut	-0.729	0.004
	Statistical ROC—No cut	-0.792	0.001
	Statistical ROC—Proportional 1%	-0.063	0.993
Modularity	No cut—Fixed 100 reads	-0.678	<0.001
	Proportional 1%—Fixed 100 reads	0.000	1
	Statistical ROC—Fixed 100 reads	0.259	0.176
	Proportional 1%—No cut	0.679	<0.001
	Statistical ROC—No cut	0.937	<0.001
	Statistical ROC—Proportional 1%	0.259	0.177
Entropy	No cut—Fixed 100 reads	1.189	<0.001
	Proportional 1%—Fixed 100 reads	-0.191	0.819
	Statistical ROC—Fixed 100 reads	-0.411	0.237
	Proportional 1%—No cut	-1.380	<0.001
	Statistical ROC—No cut	-1.600	<0.001
	Statistical ROC—Proportional 1%	-0.220	0.746

Table 3. Statistical comparison of the selected cut-off thresholds (i.e., no cut, fixed 100 reads, proportional 1%, statistical ROC) on plant species richness, Connectance, Modularity, and Entropy of interaction networks after Tukey's pairwise comparison test ($\alpha = 0.05$). Significant differences are highlighted in bold.

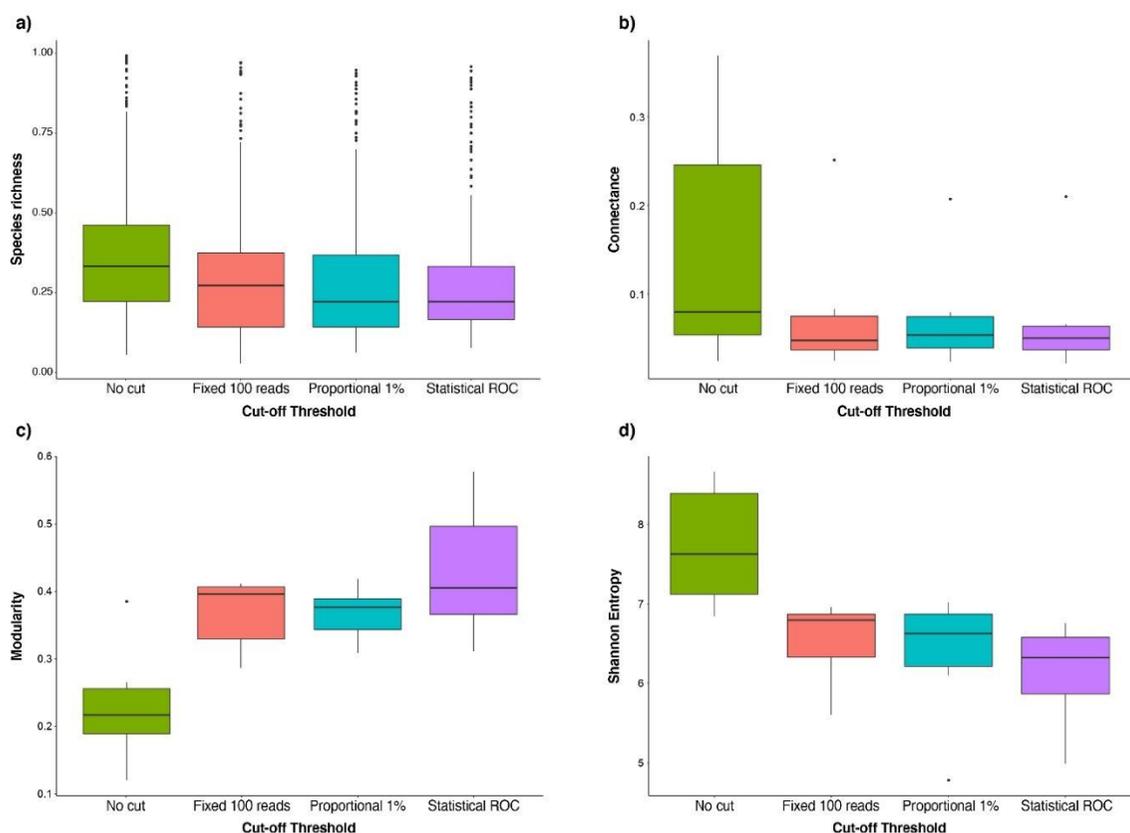


Figure 1. Changes in plant species richness (a), Connectance (b), Modularity (c), and Entropy (d) of plant–pollinator networks after using different cut-off thresholds (i.e., no cut, fixed 100 reads, proportional 1%, statistical ROC; see methods for details). Significances are reported in Table 3.

Significant effects of filtering on the network level indices were found on Connectance ($X_{32} = 11.642$, $p = 0.008$), Modularity ($X_{32} = 25.273$, $p < 0.001$), and Entropy ($X_{32} = 29.907$, $p < 0.001$). Connectance (Figure 1b, Table 3) and Entropy indices (Figure 1d, Table 3) were significantly higher, while Modularity significantly lower (Figure 1c, Table 3) in the unfiltered (i.e., no cut) compared to all the other filtering approaches. In addition, in most cases, the ROC filtering changed the network indices even more than the “proportional 1%” and “fixed 100 reads” approaches (Figure 1c,d).

The individual level index of Connectivity showed a significant effect of the interaction between the filtering approach and the normalized degree index ($X_{32} = 609.2$, $p < 0.001$). Specifically, the Connectivity was lower in the unfiltered (no cut) compared to all the other filtering types for any value of the normalized

degree (i.e., for both generalist and specialist individual pollinators; Figure 2, Table 4).

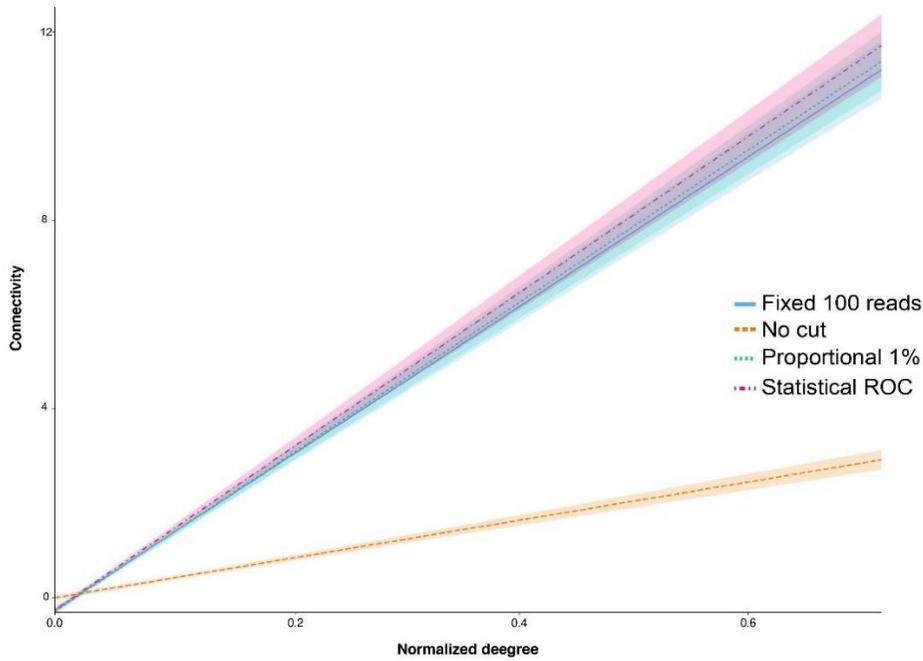


Figure 2. Response of the individual-level index of Connectivity to normalized degree in statistical interaction with the type of filtering (i.e., no cut, fixed 100 reads, proportional 1%, statistical-based ROC)

<i>Filtering Type x Normalized Degree</i>	<i>Estimated Difference</i>	<i>Significance p</i>
No cut—Fixed 100 reads	-11.896	<0.001
Proportional 1%—Fixed 100 reads	0.279	0.756
Statistical ROC—Fixed 100 reads	0.694	0.456
Proportional 1%—No cut	12.175	<0.001
Statistical ROC—No cut	12.590	<0.001
Statistical ROC—Proportional 1%	-0.414	0.662

Table 4. Comparison of Connectivity index after different filtering types in statistical interaction with normalized degree of individual pollinator (Tukey’s multiple comparison test, $\alpha = 0.05$). Significant cases are highlighted in bold.

DISCUSSION

Since its “formalization” in 2012, the DNA metabarcoding approach has revolutionized the field of biodiversity investigation, and it has even provided insights for studying biological interactions. Its application has rapidly spread and has contributed to research contexts such as microbiome [78,79], food [80,81,82], trophic ecology [83,84], and environmental DNA-based analyses [85]. In spite of its usefulness, methodological choices during the whole DNA metabarcoding pipeline and specifically the bioinformatics processing could deeply influence the obtained results and their interpretation [86,87,88]. Therefore, in this study, we attempted to evaluate the effects of the approach used to filter DNA metabarcoding outputs. Specifically, we focused on the analysis of DNA metabarcoding of pollen in the framework of plant—pollinator interactions, being aware that the outputs of our investigation could be extended to the other typologies of DNA metabarcoding-based studies. Although the issue of removing false positives and rare taxa or features is quite neglected in the scientific literature in relation to the bioinformatic pipeline (but see [28,30]), the choices made when analysing a HTS output could generate relevant effects on the community composition, species richness, and species interactions. These aspects would deeply impact the ecological outcomes of the investigated system.

The high potential of using DNA metabarcoding outputs for pollen analysis would also merit a robust bioinformatic pipeline that should be coherent and comparable among different studies. Instead, our literature overview highlighted a high heterogeneity of filtering approaches adopted to remove false positives and infrequent taxa. This is particularly appreciable even among studies that focused on similar analytical matrices (e.g., pollen from animal bodies). Surprisingly, a similarly high heterogeneity in filtering approaches emerges from studies using morphological palynology ([7] used a minimum number of 10 pollen grains, while [89,90] used a threshold of 5, and [91,92] retain species with a frequency of pollen grains above 10%, and [93], above 1%). In the case of DNA metabarcoding of pollen, our literature review showed that the proportional approach is the most recurrent, that is, to remove those molecular features or species present in the sample with reads under a certain proportion of the total reads per sample. This is quite expected, as it is an approach also well represented in other studies using DNA metabarcoding (e.g., [83,94,95]). Probably due to the

ease of calculating proportions, they bear advantages when comparing different samples or when samples would be too depauperated after a fixed raw number of reads. However, we found no concordance between different authors about the exact amount of proportion to be used as threshold, and surprisingly about the reasons justifying the choice of a particular percentage or another one (e.g., [61] used 0.1%, while [59] used 0.01%; see Table 1). It should be noticed that Peel and colleagues [53], while analysing samples of pollen prepared ad hoc with a known composition, highlighted that false positive occurred at a rate lower than 1% per sample, thus supporting this filtering strategy. On the other hand, caution should be recommended prior to generalizing the 1% threshold as a universally effective filtering practice; for samples represented by extremely high total reads count, it might be better to use a lower value. Conversely, 1% threshold can also be ineffective with almost empty samples, such as in the case of a fly that has never visited a flower but that was contaminated by airborne pollen. In such cases, it might be worth using even higher threshold values to better safeguard from misleading information.

The second most recurrent cut-off approach found in literature is based on a fixed number of read counts, used as general threshold across all samples (e.g., 50 as in [63], 100 as in [68]; see Table 1), the most frequent amount being 100 reads per sample. As reported above, with this approach, the specific value of the cut-off threshold is poorly supported by clear biological reasons. The subjectivity of authors is an important factor, and it could be a source of biases, as, for instance, studies using high threshold values would likely remove a high proportion of truly occurring taxa. For example, [56,57] observed how a threshold of 1000 reads per plant species ensures the removal of the vast majority of grass pollen species, which, however, were taxa occurring at the study areas and shall be considered true positives from airborne pollen. Therefore, low or high cutting values could have been chosen depending on the need to remove false positives but also on potential environmental contamination or infrequent species. Conversely, in other studies, the fixed cut-off value is clearly derived from sequenced negative controls. In those cases, the maximum number of reads found in blank samples is usually set as threshold (see Table 1). The assumption behind this approach is that it would allow removal of false positives exclusively originating from laboratory activities (i.e., during DNA isolation, PCR, and sequencing) [24]. However, the impact of using blanks to yield thresholds is not so clear when it comes to rare or

infrequent species that might have fewer sequencing reads than controls, and those cases would be systematically removed by this approach. Regarding this, the development of practices to retrieve negative controls for field contaminations (as hypothesized in [24]) could probably further improve the potential of this filtering method, allowing for better discrimination between species originated from field contamination and rare but truly occurring taxa.

Unfiltering seems controversial. The literature survey (Table 1) showed that nearly a quarter of studies did not report a filtering approach (based on reads count) and possibly did not filter the datasets with quantitative thresholds. However, in some of these cases, a manual filtering was used to remove the species that were not plausible in the study area [48,49,57,67], while in others, the concordance between multiple DNA barcoding markers is employed [58], thus at least partly following the recommendations to remove false positives and possibly rare taxa or features [96]. The analyses of our study clearly suggest that using a cut-off threshold for filtering the HTS output leads to significant differences compared to the unfiltered output matrix, especially in species composition, species richness and plant—pollinator interactions, impacting the ecological interpretation of the data. Our results indicated that, firstly, any of the cut-off thresholds yielded a community composition different from those obtained through unfiltered data. Moreover, filtering decreased species richness in comparison to nonfiltered data. These differences between unfiltered and filtered data could even be amplified under particular research aims. For example, in studies focusing on pollinator foraging behaviours, the unfiltering could overestimate the number of plants foraged by an animal, and it could obviously lead to an overestimation of generalism, foraging niche, and delivered ecosystem service of pollination. Another example derives from studies on honey composition, where a no-cut strategy could mislead on the purity of products, with consequences that could involve commercial issues. In our investigation, the filtering of false positives or rare taxa impacted not only the species composition and richness but also the ecological networks of species interactions. Specifically, we detected significant differences when comparing networks calculated from filtered to nonfiltered data. The implications of this network variation could potentially be very high, as, for instance, network Entropy, Connectance, Modularity, and Connectivity refer to network stability and resilience, to the ability to buffer perturbations, and to the stabilizing role of central hub species [40,97,98,99]. Thus, the higher the

difference between filtering or unfiltering strategies, the higher the potential for misleading ecological results obtained from the networks associated to each filtering type. For instance, our results showed that filtering decreased network Connectance and Entropy. This aligns well with the lower species richness per sample found in filtered datasets, and it can be explained by a decrease in network number of realized links (i.e., fewer plant species found on pollinator bodies or samples). In other words, by decreasing the numerosity of links, filtering likely yields networks with slightly higher element-specific linkage compared to nonfiltered networks. From an ecological point of view, this translates in a lower chance of overestimating generalism after filtering. Moreover, filtering increases Modularity and Connectivity of networks. This result further clarifies that removing ambiguous taxa decreases the ubiquity of links among elements, thus allowing for better emergence of ordered patterns of well-defined compartments of interactions (Modularity) and important hub species connecting them (characterized by a higher Connectivity). As a consequence, filters seem to increase the ecological reliability when describing how flower resources are used by foragers (i.e., Modularity) and the importance of certain species in contributing to interactions stability (i.e., Connectivity). In other words, unfiltering returns networks richer in links, which tend to be ubiquitously distributed among elements, with the high potential of overestimating foraging strategies and network resilience. Based on these considerations, researchers could prefer filtering their data. One exception to this would be when the role of rare plant species is targeted in the study [100]. In this case, manually checking an unfiltered dataset for unplausible taxa could limit the amount of false negatives. Moreover, integrating DNA metabarcoding data with traditional (quantitative) morphology of pollen could improve results reliability and interpretation [101]. This could possibly control for the presence of spurious information from DNA metabarcoding.

Among the filtering strategies analysed here, the statistical ROC approach appears to be the most conservative one, since it tends to yield the lowest species richness, the highest Modularity, and the lowest Connectance and Entropy. Thus, ROC-based filtering might remove not only the false positives from samples but also the infrequent species. It should be noted that ecological patterns emerging or confirmed even in a conservative framework are more likely to be trustworthy. Even if this approach has rarely been applied, to date, in the pollen DNA

metabarcoding literature (Table 1), it was specifically developed to distinguish “true signals” from “noise” [102] in molecular biology and could constitute a promising avenue for processing data of pollen DNA metabarcoding. For instance, it has been used in other DNA-based research fields [103,104], such as for eDNA where it is proved to increase the reliability of data [105]. However, it would be promising to investigate how different parametrizations of the ROC approach would impact the estimations of cutting thresholds. Because ROC is a conservative approach, it may be favoured in studies willing to highlight ecologically meaningful species composition, richness, and interactions, while sacrificing the pursuit of high species richness based on keeping elements of rarity, potential contaminants, and false positives.

CONCLUSIONS

Our survey shed light on the possible consequences of using (un)filtering strategies of pollen DNA metabarcoding data in ecological and biological research. To date, this powerful molecular tool still requires the development of shared approaches on the bioinformatic filtering of molecular features. This would improve the provision of reliable, repeatable, and comparable data. In particular, we recommend that researchers (i) always make both raw unfiltered and filtered data easily accessible, thus improving the possibility of exploring large amounts of data and, consequently, the growing rate of human knowledge in strategic research fields such as pollination ecology. The authors should (ii) apply filtering from false positives and possibly also from infrequent species, depending on research aims. Moreover, (iii) the specific type of filtering must be clearly justified under a biological perspective, evaluating the efficiency and universality of the loci selected for species identification and the consequent taxonomic resolution of molecular feature assignments. Furthermore, (iv) the specific strategy has to be decided based on whether the research aim would benefit from a conservative filtering. Without an appropriate filtering, DNA metabarcoding reads converted to presence/absence data certainly yield spurious results [30,106,107]. To avoid this, conservative approaches like the ROC filtering must be preferably adopted. Otherwise, the application of a filter either based on a percentage with a clear biological support or based on a fixed value from negative controls is possible, although greater awareness should be placed on the risk of excluding only false positives while keeping environmental contamination and infrequent species.

Supplementary Materials

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<https://www.mdpi.com/article/10.3390/d13090437/s1>

Author Contributions

Conceptualization, P.B., A.G. and N.T.; methodology, P.B. and N.T.; formal analysis, P.B. and N.T.; investigation, A.F.; data curation, N.T. and A.F.; writing—original draft preparation, N.T., P.B., A.G., M.L. and A.F.; supervision, A.G., P.B. and M.L. All authors have read and agreed to the published version of the manuscript.

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8.5 Tommasi, N., Pioltelli, E., Biella, P., Labra, M., Casiraghi, M., Galimberti, A. Effect of urbanization and its environmental stressors on the intraspecific variation of flight functional traits in two bumblebee species. bioRxiv 2021.01.29.428756; doi: doi.org/10.1101/2021.01.29.428756

In the following study chase the last intermediate aim of this thesis have been achieved by evaluating the impact of abiotic and biotic stressors related to urbanization on species dispersal functional traits. Specifically, two bumblebee species have been investigated in the metropolitan area of Milan and surrounding provinces. I personally lead all the phases of this work, from field sampling to sample processing, data analysis, as well as the writing of manuscript

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EFFECT OF URBANIZATION AND ITS ENVIRONMENTAL STRESSORS ON THE INTRASPECIFIC VARIATION OF FLIGHT FUNCTIONAL TRAITS IN TWO BUMBLEBEE SPECIES

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Keywords: Functional diversity, urban heat island, pollination, urban ecosystems, wing asymmetry, geometric morphometrics

ABSTRACT

The way urbanization shapes the intraspecific variation of pollinator functional traits is little understood. However, this topic is relevant for investigating ecosystem services and pollinator health. Here, we studied how urbanization affects the functional traits of workers in two bumblebee species (*Bombus terrestris* and *B. pascuorum*) sampled in 37 sites along a gradient of urbanization in North Italy (an area of 1800 km² including the metropolitan context of Milan and other surrounding capital districts). Namely, we investigated the role played by land use composition, configuration, temperature, flower resource abundance, and air pollutants on the variation of traits related to flight performance (i.e., body size, wing shape and size fluctuating asymmetry). These traits are relevant because they are commonly used as indicators of stress during insect development. The functional traits of the two bumblebees responded idiosyncratically to urbanization. Urban temperatures were associated with smaller wing sizes in *B. pascuorum* and with more accentuated fluctuating asymmetry of wing size in *B. terrestris*. Moreover, flower abundance correlated with bigger wings in *B. terrestris* and with less asymmetric wing sizes in *B. pascuorum*. Other traits did not vary significantly, and other urban variables

played minor effects. These patterns highlight that environmental stressors linked to urbanization negatively impact traits related to flight performance and development stability of these species with possible consequences on the pollination service they provide. Overall, this study found species-specific variation patterns in syntopic taxa, expanding our understanding about the effects of anthropic disturbance in shaping relevant functional traits of pollinator model species.

INTRODUCTION

Widespread phenomena of urbanization are driving deep changes on landscape features, their temperatures and pollutants, creating novel ecosystem conditions that impact biodiversity (Foley et al., 2005; Weng et al., 2007; Wenzel et al., 2020). Plants and animals respond to these environmental variations by shifting their distribution (Colla et al., 2012), phenology (Huchler et al., 2020), and/or shaping some morphological traits considered “functional”, i.e. relevant for their ecology, fitness and behaviour (Alberti et al., 2017; Eggenberger et al., 2019; Nooten & Rehan, 2020). In bees, trait variation due to environmental alteration could affect the efficiency of the ecosystem services they provide though impacting the way they interact with plants (Buchholz & Egerer, 2020, Biella et al., 2019). Environmental alteration could also impact pollinator development, for example by limiting the abundance of floral resources due to the increasing proportion of anthropized surfaces (Steffan-Dewenter et al., 2001). This scenario, in turn, could trigger body size declines due to less food supplied to larvae (Couvillon et al., 2010). Furthermore, landscape anthropization could change the local climate, thus altering pollinator ecology, development and foraging (Radmacher & Strohm, 2010). Specifically, the higher degree of cemented “impervious” land cover that characterizes urban areas is often associated with increasing temperatures, a phenomenon known as the “heat island effect” (Chun & Guldmann, 2018). Observations from previous studies have strengthened the hypothesis that pollinator insects could face a shift towards smaller body size as an adaptation to reduce the risk of overheating while foraging in warmer conditions (Peters et al., 2016; Gérard et al., 2018a). Considering that the worldwide steady growth of the human population is driving a dramatic urban sprawl, new insights on pollinator responses are necessary.

Previous studies investigated the morphological responses of pollinators to anthropogenic pressures, mainly focusing on body size (e.g., Chown & Gaston, 2010; Eggenberger et al., 2019; Theodorou et al., 2021). In bees, this character responds rapidly to environmental changes (Chown & Gaston, 2010), it shows little heritability, and its variation mainly depends on the amount of food received during the larval development (Couvillon et al., 2010). Bee size is positively correlated with the foraging range (Greenleaf et al., 2007). Generally, larger bees show more efficient flight performances (Harrison & Roberts, 2000), since flight muscle ratio is known to increase with body size in flying insects (Samejima & Tsubaki, 2010). Size also determines the metabolic rate and resource needs of adult imagos, with larger bees having higher metabolic rate (Kelemen et al., 2019) and thus potentially being more susceptible to shortage in floral resource availability (Couvillon & Dornhaus, 2010). However, to date, the investigation of pollinators body size variation in anthropogenic habitats yielded heterogeneous results. A recent study on bumblebees found bigger workers in cities (Theodorou et al., 2021). This study speculated that such pattern is an adaptation to longer flights for collecting resources, particularly in view of the severe green patches fragmentation of urban landscapes (Greenleaf et al., 2007). Conversely, a study by Eggenberger et al. (2019) found smaller bumblebee foragers in cities. This was interpreted as an effect of both limited local resource abundance and warmer temperature in urban areas, but the effect of these variables was not directly tested. Given these contradicting results and different interpretations, more studies are needed for clarifying the existing patterns of pollinator morphological responses to urbanization.

Morphometric studies are gaining in importance for quantifying even subtle variations in morphological traits. These variations are usually informative of stress exposure, and thus provide information about animal population health status (Adams et al., 2001). One of the advantages of using trait variation to measure stress is that changes of phenotypes are detectable before an overall decrease in population viability (Hoffmann et al., 2005). Therefore, quantifying traits variation could become an essential practice when evaluating local and landscape-level stressors. A metric that has grown in popularity is the fluctuating asymmetry (FA) (Klingenberg, 2001; Beasley et al., 2013; Alves-Silva et al., 2018), defined as the presence of small, randomly placed deviations from perfect

bilateral symmetry due to the occurrence of developmental instability, driven by exogenous environmental conditions (Klingenberg, 2015). FA differs from another type of bilateral asymmetry, the directional asymmetry (DA), that occurs when the two sides are steadily different with a predictable direction to this difference. While DA has a genetic basis and therefore could be less impacted by the environment (Palmer & Strobeck, 2003), the FA is considered a valid proxy of stress exposure to conditions that typically occur in urban environments (e.g., higher temperature and air pollutants) (Beasley et al., 2013). For instance, laboratory-based studies have demonstrated that higher CO₂ level or low temperature lead to an increase in wing FA, supporting the possible role of traffic pollutants and climatic variation in determining developmental instability (Klingenberg et al., 2001; Hoffmann, Collins & Woods., 2002). However, asymmetries could be found in wing shape and/or in wing size and they even have different responses to the same stressor type. For instance, in a recent study by Gerard et al., 2018, variations in wing size asymmetry were observed in response to thermic and parasitic stress while these same stressors caused no alteration in wing shape asymmetry level. Both wing size and shape are important functional traits in pollinators. This is because wing size is believed to be related to flight length and it influences metabolic costs (Fernandez et al., 2017; Soule et al., 2020), while shape is considered important for flight manoeuvrability (Kolliker et al., 2003, Grilli et al., 2017).

In order to characterize the effects of urbanization and of the related environmental stressors on pollinator insects, we quantified the morphological variation in two species of bumblebee (i.e., *Bombus pascuorum* and *B. terrestris*). We sampled foraging workers from populations spanned across a gradient of growing urbanization (from seminatural areas to highly urbanized sites) in Northern Italy, a region that experienced a strong anthropogenic footprint (Perini and Magliocco 2014; Salata 2017). The two species were selected as they are among the most common and widespread pollinators in Europe (Pekkarinen & Teräs, 1993; Rasmont et al., 2008), and have been largely used as model species in many studies related to the effects of urbanization or other stressors (Eggenberger et al., 2019; Theodorou et al., 2021).

We expected to find quantitative variation in bumblebee functional traits of body size and wing FA in response to several facets of urbanization. Firstly, we tested associations with increased fragmentation of green patches that is often found in

urban landscapes (Li et al., 2019). We also tested responses due to environmental stressors amplified by urbanization, such as increased temperatures (Feng et al., 2014) and pollutants (Salahodjaev 2014), and decreased floral resource abundance (Ushimaru 2014). Regarding body size we based our survey on two alternative expectations that emerged from previous studies, depending on the prevalent pressure acting on this trait. First, one could expect to observe an increase in body size if green patches fragmentation triggered an adaptation to increase foraging ranges, as suggested by (Warzecha et al., 2016). Alternatively, a reduction in body size could arise as a way to reduce the risk of overheating in warmer urban habitats (Maebe et al., 2021; Pereboom & Biesmeijer, 2003) or as a consequence of limited floral resources (Chown & Gaston, 2010). Regarding wing FA in shape and size, we expected to find increased FA in response to higher levels of biotic and abiotic stressors that are expected to occur in more urbanized landscapes, such as limited floral resources, temperature, and air pollutants.

MATERIALS AND METHODS

Study species

This study was focused on two co-occurring species of bumblebee: *Bombus terrestris* (Linnaeus 1758) and *B. pascuorum* (Scopoli 1763). Both species are pollinators common in Europe and can be easily found while foraging in different habitats (Polce et al., 2018), even in urban areas (Meeus et al. 2021, Banaszak-Cibicka, and Źmihorski 2012), including the surveyed region (personal observation of the authors). Given these characteristics, these species are reliable models to investigate responses by pollinating insects to landscape anthropization (Eggenberger et al., 2019; Theodorou et al., 2021). Using two cases of different, albeit related, model species could even clarify if the observed patterns are general or rather shaped by different life histories. The two selected species, in fact, have slightly different foraging ranges, with an estimated maximum of 449 and 758 m for *B. pascuorum* and *B. terrestris*, respectively (Knight et al., 2005). Nesting habits are also dissimilar as *B. terrestris* builds its nest in subterranean holes, while *B. pascuorum* on top of or slightly beneath the soil surface (Goulson, 2010). Another important difference is represented by their dietary regimes, since *B. pascuorum* usually have a narrower trophic niche and a preference for deep-corolla flowers (Harder, 1985) while *B. terrestris* is highly polylectic (Dafni et al., 2010, Biella et al., 2019).

Study design and sampling

Samplings were conducted at 37 sites (Fig. 1), in July 2019, between 9:00 and 12:00 only on days with sunny and windless weather conditions. The study sites were distributed within an area of about 1800 km² covering four administrative provinces, Milano, Monza e della Brianza, Lecco and Como in northern Italy. A minimum distance between sites of 1 km was imposed to avoid the non-independence of sites (Phillips et al., 2019) since it is above the maximum foraging range observed for the two species (Knight et al., 2005). Study sites were selected along a gradient of growing urbanization, ranging from areas highly dominated by seminatural hay meadows close to forest with little urban areas nearby, to sites characterized by a high degree of impervious surface (i.e., concrete, building, and asphalt). To select sampling sites, impervious surfaces were mapped in a GIS software based on a regional land use cartography (2018-DUSAF 6.0; <https://www.dati.lombardia.it/Territorio/Dusaf-6-0-Uso-del-suolo-2018/7rae-fng6>) This land use cover map is available at the scale of 1: 10000 and was developed from AGEA orthophotos and SPOT 6/7 satellite images. Sites were chosen on a visible gradient of growing impervious cover. Once identified as suitable, the land use composition in the surrounding of the candidate sampling sites was confirmed using satellite images. For each species, five to six worker specimens were captured while foraging inside a plot of about 50 m x 50 m at each site using an entomological net. After collection, the insects were stored at -80 °C until further analyses.

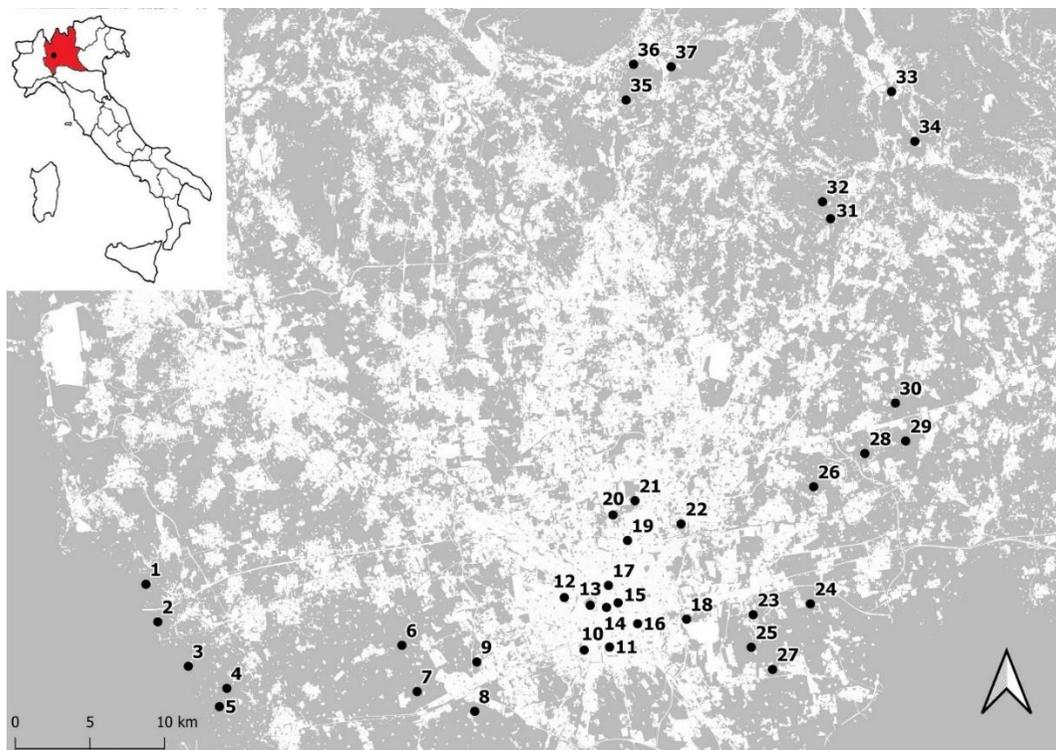


Fig.1: Map showing the distribution of the sampling sites along the urbanization gradient. White areas correspond to cemented surfaces.

Landscape and environmental variables description

The previously mentioned land use cartography was used to quantify landscape urbanization around sampling sites. Through QGIS 3.10.11, a 1 km radius buffer area was created around each site where landscape composition was evaluated arranging DUSAF original level and sub-level of land use classification into two macro categories: impervious (i.e., buildings, infrastructures, roads, and cemented surface), and seminatural land (i.e., meadows, forest and urban green spaces) (see Online resources, Additional information 2 for a list of DUSAF codes assigned to each grouping).

For each site the ratio between impervious and green land was computed to quantify the intensity of urbanization. The gradient of urbanization was also described by green habitat fragmentation, a measure of landscape configuration, that was quantified by computing the edge density (ED), as the ratio of edge length of green and seminatural patches over their total area (Wang et al., 2014). Other environmental biotic and abiotic features, possibly influenced by the

urbanization degree, were considered to test for their potential effects on altering body size and wing size/shape FA. Specifically, land surface temperature was calculated as the mean value in the period June-July using data retrieved through remote sensing imaging spectroradiometer (MODIS) MOD11A2 from the NASA database (<https://modis.gsfc.nasa.gov/data/dataproduct/mod11.php>) with a resolution of 1 km. As the two species of bumblebee studied are characterized by a life-cycle from eggs to adults of about two months (Goulson, 2010), the time frame for which these data were taken into consideration should well represent the mean temperature experienced by larvae during their development. It is important to notice that at the resolution used here it is not possible to properly describe microclimatic variation, but rather broader temperature variation covering the landscape scale and the foraging range of the two bumblebee species (Knight et al., 2005). A map reporting the variation of mean temperatures along the investigated landscape is reported in Online resources, Figure S1. Air pollution was estimated as the mean of daily concentrations of NO₂ over two months (June and July) registered by Regional agency for environmental protection (ARPA), data taken from monitoring stations located nearby our sampling sites (<https://www.arpalombardia.it/Pages/Aria/qualita-aria.aspx>). A map reporting the location of monitoring stations along the investigated landscape is provided in Online resources, Figure S1.

An expeditive estimation of floral resources at each site (i.e., the total number of flowers) was performed by using six quadrats 1 m x 1 m (covering a proportion of sampling area similar to that reported in Fisher et al., 2017) randomly placed in the flowering green spaces within or closest to the sampling area and counted the number of flowers found there (as in Ushimaru 2014). Flowers were counted considering single or composed inflorescences as units: for *Myosotis* sp., *Galium* sp., and *Capsella bursa-pastoris*, and all Asteraceae the number of inflorescences was counted. The values of the listed landscape and environmental variables in all the sampling sites are reported in Online resources, Table S1 along with histograms showing their variation along the sites (Additional information 1).

Specimens imaging and wings measurement

The forewings of all individuals were detached at the base and scanned at high resolution (i.e., 600 dpi). The obtained images were converted into TPS files using

tps-UTIL 1.74. This file format follows the standard formats for geometric morphometrics (Rohlf, 2015). TPS file can contain two or three dimensional landmark data and the information about the scale factor applied to each specimen is also provided. Once converted into TPS, images were digitised using the tps-Dig 2.31 software (Rohlf, 2015), with two-dimensional cartesian coordinates of 15 landmarks positioned at wing vein junction (Fig. 2) (as in Aytekin et al., 2007; Klingenberg et al., 2001). Bumblebees with damaged or badly worn wings were excluded from further analyses.

The analysis of landmark configuration was conducted in MorphoJ 1.07 software (Klingenberg, 2011). To remove all the effects of scale, rotation and position, a standard protocol based on a generalized least square Procrustes superimposition was applied (Klingenberg, 2011). This strategy permits to obtain a new set of superimposed landmark coordinates (i.e., 'Procrustes shape coordinates') describing the wing shape and size features. Wing size was estimated as the centroid size: i.e., the square root of the sum of squared distances from the centroid of each landmark configuration and used as a proxy of body size (hereafter "body size", as in Outomuro & Johansson, 2011 and Dellicour et al., 2017). To confirm the positive relation between centroid size and body size the inter tegular distance (IT), another measure of body size (Warzecha et al., 2016), was retrieved from a subset of 50 individuals of each species. Afterwards, the correlation between IT and centroid size was calculated (*B. terrestris* $r = 0.7$, $p < 0.001$; *B. pascuorum* $r = 0.7$, $p < 0.001$). Wing size asymmetry was computed by dividing the absolute difference between left and right centroid sizes by the mean centroid size and multiplying by 100 (Leonard et al., 2018). To estimate wing shape variation, Procrustes distances were computed for each individual (Klingenberg, 2015). These represent the measure of an individual's overall asymmetry (i.e., combining DA and FA components), obtained by taking the square root of the sum of squared distances between corresponding right and left Procrustes' coordinates (Klingenberg, 2015).

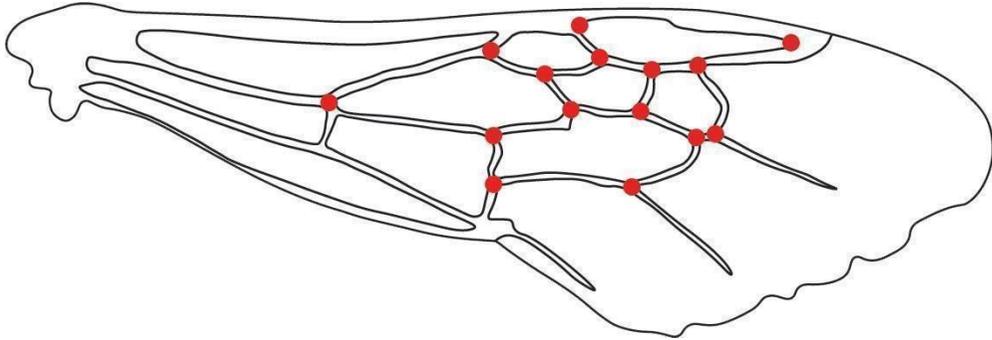


Fig.2: Right forewing of *B. terrestris* with landmark locations used in this study. Details on the formulas applied to calculate centroid size and consequently fluctuating asymmetry from these landmarks are reported in the manuscript section “Specimens imaging and wings measurement” and in the references within.

Statistical analysis

According to the protocol outlined in Klingenberg 2015, we first estimated the entity of the measurement error because the levels of asymmetry in bilateral traits are subtle and it could possibly cause considerable variation in the assessment of asymmetry levels. This was performed by double-scanning the wings and digitizing their landmarks for a subset of 40 specimens, with the Procrustes ANOVA in MorphoJ (Klingenberg 2001, Klingenberg 2015) in order to evaluate the measurement error. Afterwards, following Costa et al. (2015), to isolate the FA component, we calculated the amount of directional asymmetry (DA) and tested its entity again with the Procrustes ANOVA in MorphoJ considering all the measured specimens in a single analysis. Only if DA occurred significantly, the individual asymmetry measures were corrected by subtracting the mean DA, thus isolating the FA component as in Costa et al. (2015).

To investigate the relationship between morphological traits and covariates, linear mixed models were used. The responses of the two species were assessed separately. In all the models, the ratio between impervious and seminatural surfaces was initially included as a predictor with the other variables. However, Variance inflation factor (VIF) criteria was used to assess the absence of collinearity among model variables and it indicated that the ratio between impervious and seminatural surfaces was highly collinear with the other variables, see also the correlation matrix reported in Online resources, Table S2. Thus, we decided to exclude the ratio between impervious and seminatural surfaces from

subsequent models. The other variables, describing landscape configuration, biotic, and abiotic features, as well as the interaction between all these variables, were included in the models following the ecological expectations of our hypothesis. Specifically, changes in body size were evaluated in response to the edge density of green area, temperature, and floral resource availability because they could directly influence body size, with bigger sizes in increasingly fragmented green areas, and/or with more flower resources, and/or less temperatures (Warzecha et al., 2016; Pereboom & Biesmeijer, 2003; Chown & Gaston, 2010). Concerning wing FA, the temperature, concentration of NO₂, and flower resources limitation were included in the models following our hypothesis that they could be stressors expected to increase asymmetry (Hoffmann, Collins & Woods, 2002; Klingenberg et al., 2001; Leonard et al., 2018) Sampling site was included as a random effect in all the models. A backward stepwise model selection based on AIC was used to remove variables and their combinations that did not improve the model fit and thus to obtain reliable final models (Zuur et al., 2009). In order to improve the fit between the predictors and the response variable, mathematical transformations were applied on some of the covariates as reported in Table 1. All the analyses were performed using R (version 3.6.1; R CoreTeam 2019).

RESULTS

After excluding queen, males, and specimens presenting damaged wings, 179 *B. pascuorum* (mean per site = 4.8 ± 0.4) and 169 *B. terrestris* (mean per site = 4.5 ± 0.3) were subjected to morphometric analyses.

The measurement error was negligible because it was not significant for wing size (df = 79, F = 2.67 p = 0.4578, R² = 0.0009) and shape (df = 2054, F = 0.51, p = 0.9976, R² = 0.07). Different patterns of size variation were found in the two bumblebee species. *B. terrestris* body size was found to increase in response to floral resource abundance (Fig. 3a, Table1) while *B. pascuorum* body size decreased in response to the increasing temperature (Fig. 3 b, Table 1).

Concerning wing asymmetry, both species showed a significant level of shape DA (*B. pascuorum* df = 26, F = 4.66; p < 0.0001, R² = 0.008; *B. terrestris* df = 26, F = 5.60; p < 0.0001, R² = 0.009), while size DA was statistically significant only in *B. pascuorum* (df = 1, F = 29.77; p < 0.0001, R² = 0.0007; in *B. terrestris* df = 1, F = 0.51; p = 0.4779, R² = 0.000008). Measures of FA were then obtained by

correcting for directional components.

B. terrestris size FA was positively correlated to temperature (Fig. 4 a; Table 1) while *B. pascuorum* size FA decreased with higher floral resource availability (Fig 4 b; Table 1). None of the predictor variables (i.e. temperature, NO₂, resource abundance, and the interaction among these variables) showed a significant effect on wing shape asymmetry in both bumblebee species (output of non-significant regression models are available in Online resources, Table S3).

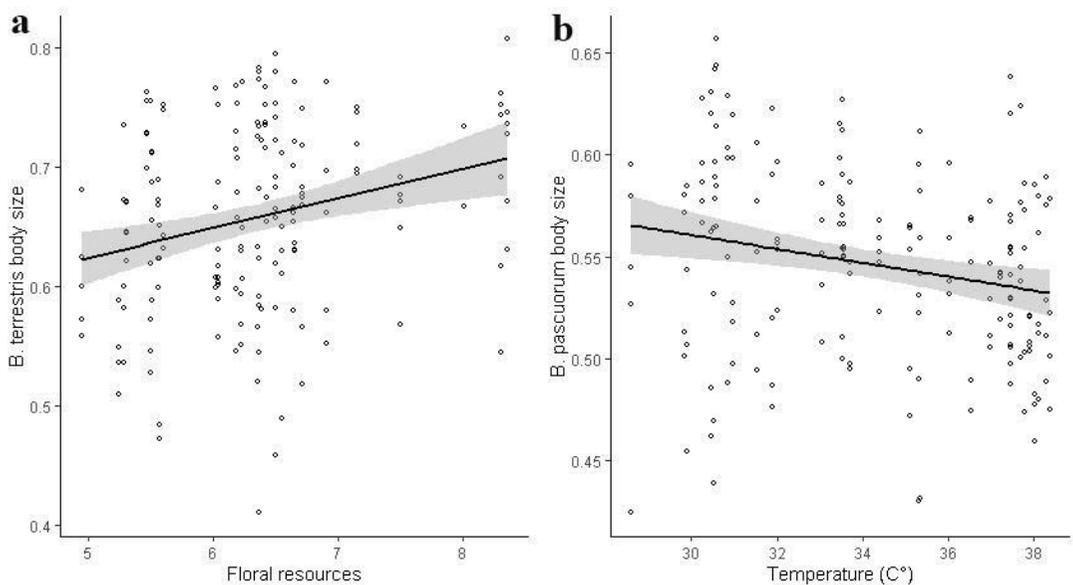


Fig. 3: Body size variations as a function of (a) Floral resource abundance in *B.terrestris* and (b) Summer temperature in *B. pascuorum*. The black line and grey areas indicate the relationship and its confidence intervals as estimated with Linear mixed models, see methods for further details.

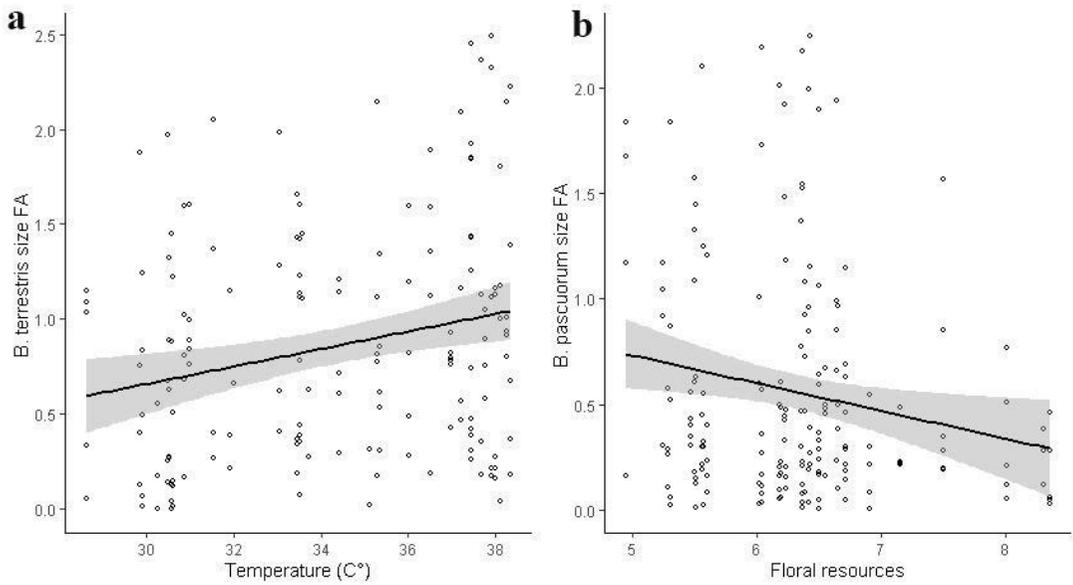


Fig. 4: Variations in wing size Fluctuating Asymmetry (FA) as a function of (a) Summer temperature in *B. terrestris* and (b) Floral resource abundance in *B. pascuorum*. The black line and grey areas indicate the relationship and its confidence intervals as estimated with Linear mixed models, see methods for further details.

Table 1: Output of Linear mixed models of body size (N= 348) and fluctuating size asymmetry (size FA) (N= 347) of each species as a function of biotic and abiotic covariates of urbanization, with site identity as random factor. Final models were selected through backward stepwise selection using AIC criterion. Δ AIC reports the difference in AIC values between full and final models. β i: regression coefficient; χ^2 : chi square values; df: degrees of freedom. Models and results of shape FA are reported in Online resources, Table S3 as they were non-significant.

Species	Response variable	Full model covariates	Final model covariates	Δ AIC	Bi	χ^2 ; df	p value
<i>Bombus terrestris</i>	Body size	Temperature T Edge density ED log (Floral resources FL) Interaction T x ED x FL	log (Floral resources)	16.5	0.025	6.610;1	0.010
<i>B. pascuorum</i>	Body size	Temperature T Edge density ED log (Floral resources FL) Interaction T x ED x FL	Temperature	17.9	-0.003	7.403;1	0.006
<i>B. terrestris</i>	Size FA	Temperature T log (Floral resources FL) NO ₂ N Interaction Tx FL x N	Temperature	23.5	0.052	7.183	0.007
<i>B. pascuorum</i>	Size FA	Temperature T log (Floral resources FL) NO ₂ N Interaction Tx FL x N	log (Floral resources)	30.3	-0.161	6.118;1	0.013

DISCUSSION

In this study, we quantified the spatial intraspecific variation of functional traits in two common bumblebee species, to infer the possible alteration of their ecological features, such as the dispersion ability, with consequences on different aspects of their life history.

We focused on the morphological variations of *B. terrestris* and *B. pascuorum* along an urbanization gradient within an area of 1800 km² including the metropolitan context of Milan and other surrounding capital districts. Our results highlighted some correlations between stressors related to urbanization, and traits as body size and wing size asymmetry. Specifically, the landscape temperature and the abundance of floral resources, two environmental features influenced by the degree of urbanization (See online resources Table S1, Additional information 1, and Table S2), emerged as candidate drivers of intraspecific variation of body size across bumblebee populations, acting differently on the two investigated species. Foragers of *B. pascuorum* showed a shift towards smaller body size in response to increasing temperature, a condition often associated with deeply urbanized landscapes, generally referred to as the heat island effect. Although a similar pattern of body size reduction in urban bumblebees has previously been reported by Eggenberger et al. (2019), they did not evaluate the effect of temperature but only proposed it as a possible driver of the observed decrease of body size. This relationship has been investigated on multiple historical series collections-based and experimental studies that revealed how higher environmental temperatures represent a driver of body size reduction in different species of bees (e.g., Nooten & Rehan, 2020; Theodorou et al., 2021). Higher temperature accelerates larval development, which likely results in smaller adults (Sibly & Atkinson, 1994). Furthermore, smaller sizes in warmer areas could also be a strategy for reducing overheating risks while foraging, due to an increased convective heat loss in smaller bees (de Farias-Silva & Freitas, 2020). Functionally, smaller foragers could travel shorter foraging distances (Greenleaf et al., 2007) and could also load less pollen and nectar (Goulson et al., 2002). As a consequence, the shift towards smaller body size in *B. pascuorum* could imply that it will pollinate less plants or handle flowers less efficiently (Földesi et al., 2020), a concerning aspect in view of colony provision and pollination. It is not known if microclimatic conditions could mitigate the effects we observed, as we used

temperature measured at a broader scale. Furthermore, it is important to underline that other landscape variables could act synergically with urban temperature. However, these synergic effects could be difficult to disentangle in the field, due to the high correlation of these variables with the amount of impervious land. Further investigations considering microclimatic variations (e.g., by using data loggers at each sampling site) and field experiments pointing at cause-effect relationship between temperature and body size will be required to exclude the possible role of other urban related stressors. The role of different categories of impervious surface (i.e., concrete, building, and asphalt) in contributing to temperature increase should also be addressed in future research, to better inform mitigation strategies in urban contexts.

Body size reduction was also previously explained by the decrease in floral resource abundance in urban landscapes, possibly as a consequence of reduction of green areas (Merckx et al., 2018; Eggenberger et al., 2019). According to this evidence, we found a correlation between *B. terrestris* body size and the abundance of flower resources, with larger individuals observed where more food is potentially available. This is in accordance with the observation that adult size is strictly correlated with the amount of food received during larval development (Couvillon & Dornhaus 2009). However, this trend seems to be not clearly confirmed by *B. pascuorum* probably due to a possibly higher flower specialization of this species (Harder, 1985). Indeed, the narrower diet of *B. pascuorum* could result in the inability of this species in exploiting abundant flowering plant species that do not represent its host plants. It seems a fruitful avenue of future research to investigate other important features such as the nutritional quality of available resources, their diversity, and changes along landscape variation (Vaudo et al., 2015; Vaudo et al., 2016). Here, we considered floral resource abundance at the local scale. In spite of this limitation, we could observe some relationships between traits and flower resources. However, additional data will further illuminate how resources shape urban pollinator traits.

Despite *B. pascuorum* and *B. terrestris* belong to the same genus, they showed a different susceptibility towards the investigated stressors. This suggests that different responses are likely to come from different habits and behaviour. Idiosyncratic responses were also observed in other bumblebee species, where body size decreased over warming decades, but others responded in the opposite

way (Gérard et al., 2020). In our study the invariant size of *B. terrestris* in warmer conditions could be explained by its higher heat tolerance (Martinet et al., 2020). In addition, *B. terrestris* nests further underground compared to *B. pascuorum*, and it might be less exposed to warm air temperatures during larval development. These aspects strengthen the hypothesis that temperature could be a major determinant of pollinator size reduction in cities because they affected the body size of the more temperature-sensitive species, but not the heat-tolerant one. These idiosyncratic species-specific responses are very relevant for understanding the potential mechanism of intraspecific trait variation associated with urbanization and supports the need to consider a wider panel of species in this kind of studies.

Regarding wing asymmetry we found that size FA was positively correlated with increased temperatures in *B. terrestris*. Variation in both wing size and wing shape asymmetry was observed in other insect taxa and the effect of temperature was previously investigated under controlled laboratory conditions (Mpho et al., 2002). Studies associated the increased wing size and shape FA to environmental stressors, indicating that impairment of developmental processes might take place (e.g., Klingenberg et al., 2001; Kerr et al., 2013). The absence of variation in shape asymmetry registered for both the bumblebee species could confirm the results from other studies that have indicated shape variation as less susceptible to stressors than size asymmetry (e.g., Gérard et al., 2018b). Importantly, floral diet could represent a possible mitigation of environmental stressors during development (Archer et al., 2014). Here, this view is supported by the negative correlation found between resource abundance and wing size FA, although only in *B. pascuorum*.

Flight performance largely depends on body size and is also affected by asymmetries in shape and size between wings (Grilli et al., 2017; Soule et al., 2020). Variation in these traits does not only show developmental instability, but also has deep ecological implications. Indeed, body size is determinant in predicting dispersal ability of insects, thus determining their foraging range (Greenleaf et al. 2007). Similarly, wing size FA impacts the management of lengthy flights (Fernandez et al., 2017, Soule et al., 2020), while wing shape FA is often associated with flight maneuverability. The combination of these morphological changes could deeply impact flight performance, flight length in time and space, and consequently bumblebee foraging (Kenna, Pawar & Gill., 2021), with potential

consequences on their pollination efficiency. However, an important aspect to consider is that wing size and its asymmetry could even determine behavioural changes. For example, insects could increase visitation rates at closer distances to colonies, and even spend a higher time on the available resources instead of flying at a broader distance (Andrieu et al., 2009). Such changes, albeit difficult to quantify in the field, could merit further investigation when trying to forecast the impact of functional trait changes in response to urbanization.

CONCLUSIONS

This study suggests that the environmental changes associated with urbanization could affect different functional traits of pollinators, and that their impact occurs heterogeneously on different insect species. Eventually, as the studied traits are often involved in flying abilities, these responses could bring to the alarming outcome of decreased foraging efficiency and pollination effectiveness. Furthermore, the different responses to the same stressor among the two bumblebees underline the necessity for future studies to consider a wider panel of taxa instead of single model species in order to draw conclusions that could be applied to the whole pollinator insects community.

From a conservation perspective, the comprehension of how pollinators cope with the challenging conditions occurring in novel anthropogenic habitats, plays a key role in informing suitable policy efforts to conserve their biodiversity and the ecosystem service they provide. In the future, cities are predicted to expand constantly and thus designing of urban landscapes will become a fundamental step for achieving sustainability outcomes. The pollinator-friendly design and management of urban green spaces will possibly create suitable conditions for pollinators and thus for the ecosystem services they provide (Guenat et al., 2019; Tommasi et al., 2021). At the same time, urban forestry and greenery practices (e.g., plantation of street and residential trees and the creation of urban greenbelts or greenways) could represent a valid solution to mitigate stressful conditions related to the urban environment, such as the lack of floral resources and the heat island effect (Chun & Guldmann, 2018) that here were found to influence functional traits.

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Declarations:

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Conflicts of interest/Competing interests: The authors declare that no competing interests exist.

Ethics approval: Sampling permits were obtained when needed from local authorities

Availability of data and material: All relevant data are within the paper or stored in a public repository (<https://doi.org/10.6084/m9.figshare.13637594>).

Author contribution

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

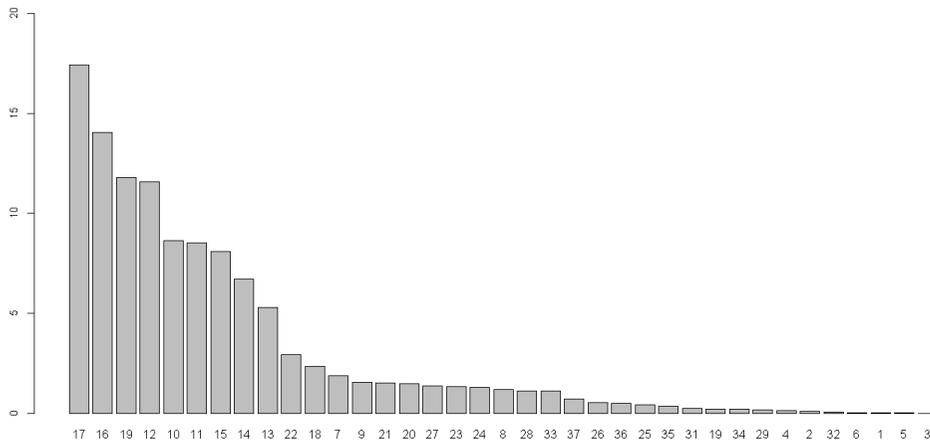
Online resources

Table S1 - Table of variables distribution among sampling sites

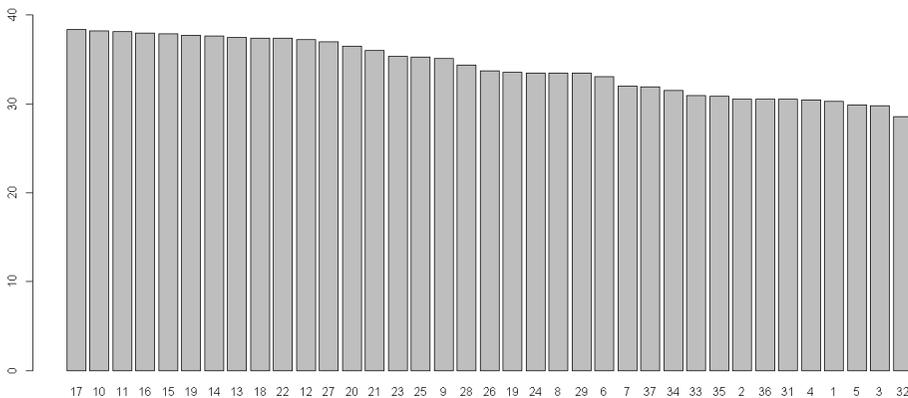
SITE CODE	Impervious/semi natural surfaces	Edge density	Min C°	Average C°	Max C°	Min NO2 µg/m3	Average NO2 µg/m3	Max NO2 µg/m3	Floral resources
1	0.0	0.040	28.6	30.3	32.7	7.1	22.44	41.100	510
2	0.1	0.045	28.4	30.6	33.2	10.4	26.87	49.800	823
3	0.0	0.020	27.2	29.9	32.8	2.0	15.25	32.800	198
4	0.1	0.055	28.6	30.5	32.2	2.0	15.25	32.800	1001
5	0.0	0.043	27.7	29.9	31.9	2.0	15.25	32.800	141
6	0.0	0.069	30.9	33.1	35.5	8.0	18.17	42.000	490
7	1.9	0.088	29.6	32.0	33.8	10.7	23.82	51.900	622
8	1.2	0.069	31.4	33.5	35.5	10.7	23.82	51.900	4049

9	1.5	0.077	32.8	35.1	37.5	19.0	26.12	31.000	3020
10	8.6	0.093	36.2	38.3	40.7	16.1	30.77	52.400	662
11	8.5	0.063	36.1	38.1	40.5	16.1	30.77	52.400	190
12	11.6	0.073	34.9	37.2	39.5	16.1	30.77	52.400	583
13	5.3	0.038	35.2	37.5	40.1	16.1	30.77	52.400	583
14	6.7	0.051	35.2	37.7	40.3	16.1	30.77	52.400	270
15	8.1	0.053	35.8	37.9	40.5	16.1	30.77	52.400	244
16	14.1	0.065	36.3	38.0	39.9	16.1	30.77	52.400	577
17	17.4	0.081	35.6	38.4	40.8	16.1	30.77	52.400	410
18	2.3	0.044	35.2	37.5	39.3	16.1	30.77	52.400	1273
19	11.8	0.083	36.1	37.8	39.5	21.3	40.89	57.600	826
19	0.2	0.071	31.4	33.6	34.9	10.0	19.29	38.000	420
20	1.5	0.046	34.9	36.5	37.9	21.6	39.94	53.500	248
21	1.5	0.023	34.2	36.0	38.0	21.3	40.89	57.600	201
22	2.9	0.086	35.3	37.4	39.5	31.0	38.19	55.000	1805
23	1.3	0.069	33.9	35.4	36.9	12.0	17.62	29.000	420
24	1.3	0.057	31.7	33.5	35.1	12.0	17.62	29.000	664
25	0.4	0.096	33.1	35.3	36.8	12.0	17.62	29.000	612
26	0.5	0.036	31.5	33.7	35.6	15.0	22.69	28.000	592
27	1.4	0.069	33.9	37.0	39.0	14.0	17.5	25.000	4270
28	1.1	0.085	32.5	34.4	35.8	19.1	32.06	49.700	765
29	0.1	0.083	31.6	33.5	34.8	19.1	32.06	49.700	261
31	0.2	0.063	28.6	30.5	33.0	6.0	10.15	17.000	487
32	0.1	0.035	27.2	28.6	31.2	6.0	10.15	17.000	703
33	1.1	0.089	28.5	31.0	33.0	6.4	12.81	22.300	504
34	0.2	0.053	29.2	31.5	33.3	1.5	7.84	15.400	262
35	0.3	0.056	29.3	30.9	32.2	6.3	14.22	23.900	238
36	0.5	0.075	28.0	30.6	32.6	6.3	14.22	23.900	774
37	0.7	0.061	29.9	31.9	35.2	5.0	9.24	12.000	487

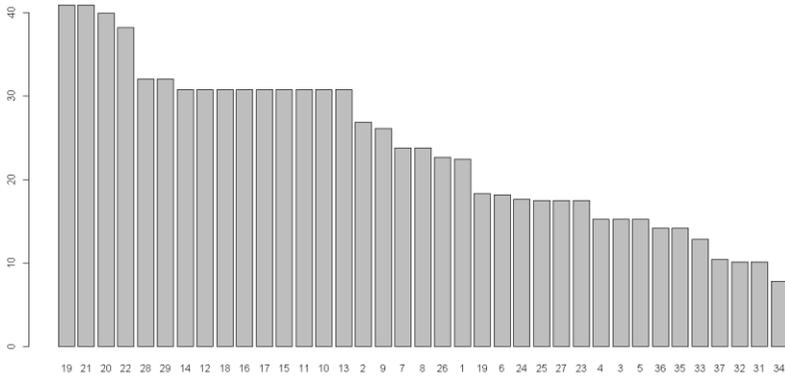
Additional information 1 - Histograms representing variables distribution among sampling sites



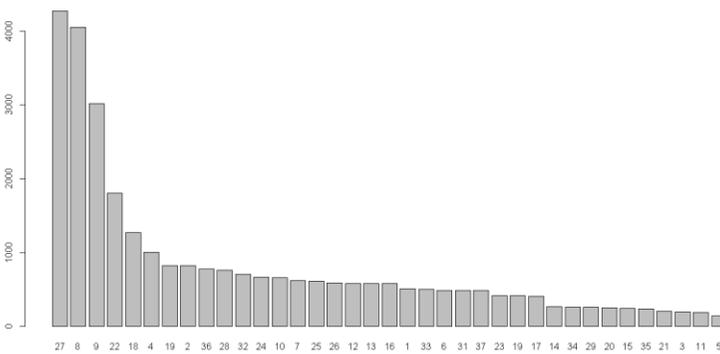
Impervious/semi natural ratio along the sampling sites of the urbanization gradient (range 0 -17.4)



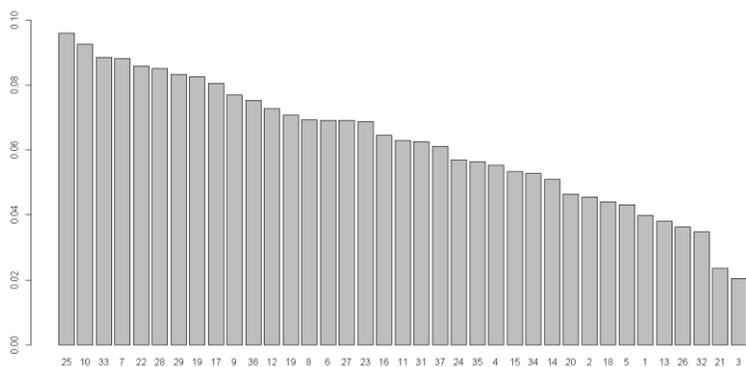
Distribution of temperature (C°) values along the sampling sites of the urbanization gradient (mean range 28.6° – 38.4°)



Distribution of NO₂ (µg/m³) values along the sampling sites constituting the urbanization gradient (mean range 23.7 µg/m³ -40.8 µg/m³)



Distribution of Floral resource availability (estimated number of flowers in standardized units of space) along sampling sites constituting the urbanization gradient (range 141-4270)



Distribution of Edge density values (measure of green patches fragmentation) along the sampling sites constituting the urbanization gradient

Table S2 - Correlation matrix between variables

	NO2	Edge density	Temperature (C°)	Impervious/natural surfaces	Floral resources
NO ₂	1.00	0.09	0.75	0.64	0.02
Edge density	0.09	1.00	0.27	0.28	0.35
Temperature (C°)	0.75	0.27	1.00	0.84	0.12
Impervious/natural surfaces	0.64	0.28	0.84	1.00	0.03
Floral resources	0.02	0.35	0.12	0.03	1.00

Figure S1 - Map of mean temperature and NO₂ sampling points

The following map shows the mean temperature in the may-july period. The location from where NO₂ values were recovered from the same period are also reported with a triangle. Sampling site locations are pointed by dots

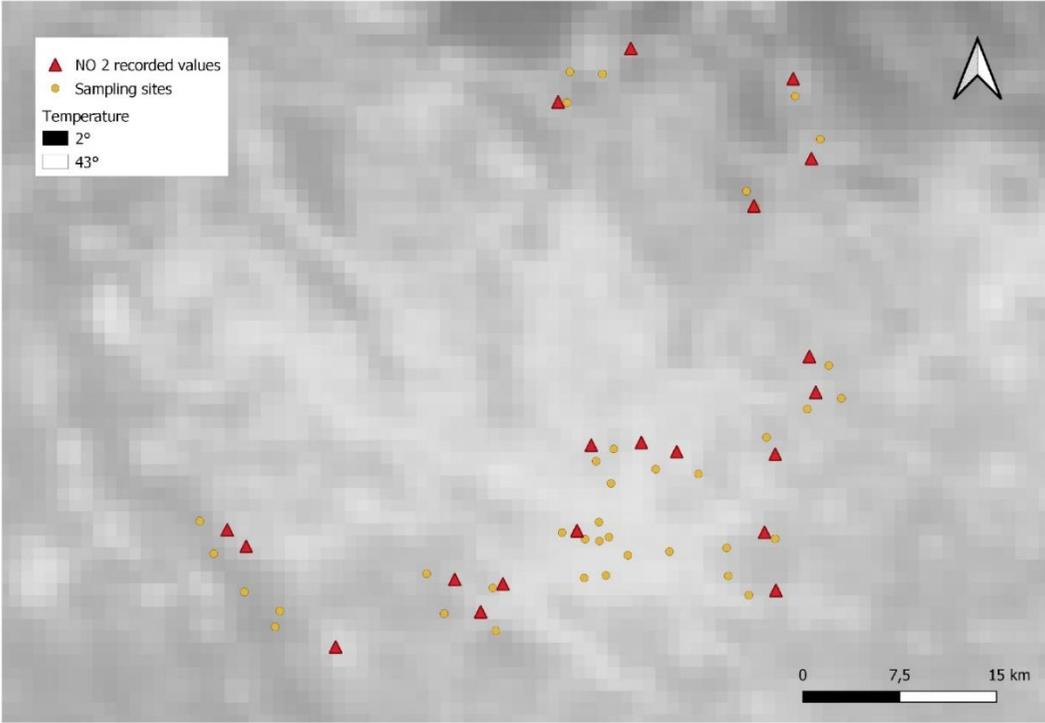


Table S3 - Complete regression models outputs

Output of Linear mixed models of body size (N= 348) and fluctuating size asymmetry (size FA) (N= 347) of each species as a function of biotic and abiotic covariates of urbanization, with site identity as random factor. Final models were selected through backward stepwise selection using AIC criterion. Δ AIC reports the difference in AIC values between full and final models. β_i : regression coefficient; χ^2 : chi square values; df: degrees of freedom.

Species	Response variable	Full model covariates	Final model covariates	Δ AIC	β_i	χ^2 ; df	p value
<i>Bombus terrestris</i>	Body size	Temperature	log (Floral resources)	16.5	0.025	6.610;1	0.010
		Edge density log (Floral resources) Variables interaction (1 Site)	(1 Site)				
<i>B. pascuorum</i>	Body size	Temperature	Temperature	17.9	-0.003	7.403;1	0.006
		Edge density log (Floral resources) Variables interaction (1 Site)	(1 Site)				
<i>B. terrestris</i>	Size FA	Temperature	Temperature	23.5	0.052	7.183	0.007
		log (Floral resources) NO ₂ Variables interaction (1 Site)	(1 Site)				
<i>B. pascuorum</i>	Size FA	Temperature	log (Floral resources)	30.3	-0.161	6.118;1	0.013
		log (Floral resources) NO ₂ Variables interaction (1 Site)	(1 Site)				
<i>B. terrestris</i>	Shape FA	Temperature				0.819;1	0.366
		log (Floral resources)				1.088;1	0.297
		NO ₂				0.774;1	0.379
		Variables interaction (1 Site)				0.689;1	0.407

Species	Response variable	Full model covariates	Final model covariates	ΔAIC	B_i	$\chi^2; df$	p value
<i>B. pascuorum</i>	Shape FA	Temperature				2.508;1	0.113
		log (Floral resources)				0.023;1	0.881
		NO ₂				0.624;1	0.429
		Variables interaction (1 Site)				0.675;1	0.411

Additional information 2 - Description of DUSAF levels categorised as “Impervious” and “Semi Natural”

List of Level 3 and 4 DUSAF codes Categorized as “Impervious”:

“111”, “1121”, “1122”, “1123”, “1211”, “1212”, “122”, “124”, “131”, “132”, “133”, “134”, “1421”, “1422”, “1423”

and “Seminatural”: “141”, “224”, “231”, “311”, “314”, “322”, “324”, “331”, “332”, “411”.

Full explanation of codes is available at:

https://www.cartografia.regione.lombardia.it/metadata/Dusaf/doc/Legenda_DUSAF_2018_6_0.pdf

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9. CONCLUSIONS

Since urbanization and agricultural intensification, the two major drivers of land use intensification causing pollinators decline, are increasing worldwide (Montero-Castaño & Vila, 2012), a broader and integrative comprehension of these phenomena is required to mitigate the pressure on ecosystems and related services. In this framework, the results obtained in this PhD project provide novel insights on how pollinator insects are affected by landscape composition, configuration and by the stressors associated to these land use features. Moreover, the studies here presented successfully improved the knowledge about pollinators diversity and ecology from countries largely neglected by this research field (e.g., the Sub-Saharan Africa), and that are facing rapid development of urban and/or agricultural activities. Furthermore, technical advances in the field of pollinator studies have been achieved through the adoption of modern molecular technologies (e.g., High Throughput Sequencing). In this conclusive chapter the main outputs of this PhD thesis are summarized along with future perspectives and take-home messages.

Effects of Landscape and local scale features on pollinators

Overall, the results obtained in the studies presented clearly identify the negative effects of the main drivers of land use intensification, urbanization, and agricultural activities expansion, on multiple aspects related to pollinator communities. Changes in landscape composition, such as the increasing proportion of impervious surfaces (e.g., streets, buildings, and infrastructures) and cultivated land, have been proved to reduce pollinator species richness and their abundance (Chapters 8.1 and 8.3). Interestingly, lower disturbance degrees (i.e., proportion of impervious surfaces lower than 50%) have been found to increase pollinator abundance and species richness (Chapters 8.2 and 8.3). Similarly, landscape configuration, specifically the increasing fragmentation of green habitats due to urbanization, similarly reduces pollinator abundance (Chapter 8.3). However, also in this case, a moderate habitat fragmentation degree has been found to support higher pollinator richness and abundance (Chapters 8.2 and 8.3).

These patterns seem to confirm what already proposed by Wenzel and colleagues

(2020). Intermediate disturbance conditions may benefit pollinator insects by favouring the creation of more heterogeneous habitat, or because diversity is reduced when competitive exclusion occur in more stable conditions, and conversely only the more tolerant species are maintained when disturbance is too high (Coulin et al.,2019). As a major consequence, the pollinator biodiversity loss occurring in response to these changes of landscape composition and configuration could greatly impact on the pollination service provided by insects. Indeed, it is known that pollinator biodiversity is directly related to the pollination efficiency (Garibaldi et al., 2017).

Landscape composition and configuration have also been found to shape properties of the interaction network between insects and plants. As urbanization and agricultural intensification increases, a simplification in the variety of collected resources have been observed, with a consequent increase in the competition among pollinator individuals (Chapters 8.1 and 8.3). Similarly, also green habitat fragmentation has been proved to reduce the complexity of the observed plant-pollinator interactions. This trend of simplification, in turns, usually subtends a lower functional redundancy of insects, thus a lower stability of the plant-pollinator interaction system (Kaiser-Bunbury et al., 2017). However, idiosyncratic responses have been highlighted in different species, supporting the need to promote target investigations on those species that mainly participate to interaction network stability in specific habitats. While all these changes in plant-pollinator interaction features may alter the pollination service efficiency, this linkage has poorly been investigated before. This thesis work addressed this issue without highlighting a direct link between plant-pollinator interaction features and pollination efficiency. However, the results here reported further support the direct relationship between pollinator biodiversity and pollination efficiency.

Local scale biotic (e.g., plant and pollinator species richness and abundance) and abiotic features (microclimate conditions) can also deeply influence pollinator community features.

In the results presented in this thesis, the local scale temperature variation, that is indirectly influenced by landscape composition, not only influence pollinator diversity, but also morphological traits related to their dispersal ability (Chapters 8.3 and 8.5). While these changes may ensure the investigated species to cope

with the climatic variation of the urban habitats, the functional consequences of these possible adaptations are still unexplored. Future investigations should aim at addressing this issue, as well as increasing the panel of investigated species to provide a wider overview of these findings.

Biotic local scale conditions, such as the abundance or richness of floral resources, have been proved to benefit pollinators biodiversity, also reversing the decreasing trend posed by land use intensification. Similarly, plant-pollinator interactions have been proved to be shaped by local floral resource features, but also by local aspects of the pollinator communities. For example, high pollinator abundance increased the interaction network stability, possibly because of competition dynamics. Beyond the local availability of floral resources, their variations in terms of nutritional features, also considering the response to land use changes should be considered. This relationship has preliminarily been highlighted in this thesis (Chapter 8.3); however, future research should investigate a wider panel of nutritional compounds and assess how deeply the variation in resource nutritional profiles could impact pollinator foraging choice and health.

Towards a pollinator friendly landscape management

Land use planning policies can have a major impact in determining pollinator community health, diversity, and functionality.

The main finding of the present PhD thesis is that the maintenance and the creation of green areas in urban matrices should be encouraged to contrast or at least mitigate the negative impact of land use intensification and the related stressors on pollinator communities and provided services. By reducing the proportion of impervious surfaces, this kind of urban management strategy could support species richness and abundance in urban areas by creating new nesting sites and more foraging opportunities. Moreover, a higher abundance in green spaces is supposed to mitigate microclimate variations related to landscape composition, as the heat islands, and the effects that these stressors have on biodiversity and functional traits. Similarly, the maintenance of semi natural habitats should be promoted in agricultural landscapes, as in turn this could directly benefit agricultural productivity.

Not only the availability of green spaces should be promoted, but also the interconnection among them. Indeed, this may prevent biodiversity loss and facilitate pollinators dispersal among patches increasing their possibility to find proper food resources. Therefore, the promotion of favourable small green patches acting as steppingstones and the creation of ecological corridors to facilitate exchanges among wider green areas should be considered when developing land use planning policies.

The results achieved also clearly support the adoption of nature-based solutions that can directly benefit pollinator by improving resource availability, for example through the creation of hedgerows and flower strips both in urban and agricultural landscapes. According to what previously highlighted in Garibaldi et al. (2014) this is necessary to directly increase the availability and diversity of floral resources and is proved to promote pollinator abundance and species richness.

Nature-based solutions and new land use policies should be fostered to create pollinator-friendly conditions, even in the more anthropized landscapes.

Technical advances in the study of plant-pollinator interactions

In the framework of this PhD thesis also technical/methodological aspects have been elucidated, especially concerning the DNA metabarcoding assessment of plant-pollinator interactions. Although its use in the analysis of mutualistic interactions cannot be considered a novelty, multiple open issues raised from the study cases here presented. A clear lack of shared methodology emerged in different analytical phases of the pollen DNA-based identification (e.g., pollen recovery, DNA barcode marker). Especially, the ecological consequences of filtering raw data through different strategies have been clearly disentangled in this PhD project. This has been useful to set a milestone necessary to improve the standardization of analytical approaches, and thus increase the possibility to compare data from different studies. Although clearly focused on the use of DNA metabarcoding for the analysis of plant-pollinator interactions, similar issues also affect all the other fields of application of this approach, such as the analysis of pollinator microbiome, or even studies not necessarily related to pollinators.

The efficiency of this approach has also been demonstrated through its application in previously under investigated contexts, such as Sub-Saharan Africa. In this context, the availability of DNA reference sequences is essential to provide

a reliable and supported taxonomic identification of pollen resources. However, in the studies included in this thesis, this drawback has been successfully overcome through the production and publication of reference sequences for many flowering plant species occurring at the study sites and not available in public repositories.

Take-home messages

The global population is progressively becoming more conscious of the problem arising from the ongoing pollinator decline (Hall et al., 2020) and measures to face this issue have already been taken. For example, the European green deal (Tamma et al., 2019), the new growth strategy of European Union that aims at improving citizen well-being and making the country climate neutral, aspires at halting and reversing the pollinator insects decline in the next years (Arpaia et al., 2021). Moreover, the European Union is acting on pollinators conservation through specific actions, such as the EU pollinators initiative (2020). At the international and national levels, the Food and Agriculture Organization of the United Nations (FAO, www.fao.org/pollination/en/) is also promoting multiple initiatives to face this problem through active measures for pollinator community management and by improving people awareness on the consequences of pollinator loss (e.g., the World Bee Day).

The results presented and discussed in this PhD thesis clearly fit with such international efforts towards pollinators conservation. The multiple claims for a pollinator-friendly management of anthropic landscapes, also supported by the results here presented, can be successfully employed to achieve global conservation and sustainability goals.

Putting into practice all the possible interventions to protect and promote pollinator health and functionality is necessary not only to preserve the intrinsic values of biodiversity, but also because of the close interlinks between pollinators and food security, and more in general human health.

In the next future, policy makers should improve the integration of pollinator research outcomes into management actions, recognising what already proposed by the “One health” concept (<https://www.cdc.gov/onehealth/basics/index.html>) so that the health of people, biodiversity and ecosystems are closely connected. In

this context, interventions for safeguarding public health issues cannot be exempted from considering the health and conservation of ecosystems.

10. 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. Biella, P., Tommasi, N., Akter, A., Guzzetti, L., Klecka, J., Sandionigi, A., ... & Galimberti, A. (2019). Foraging strategies are maintained despite workforce reduction: A multidisciplinary survey on the pollen collected by a social pollinator. *PloS One*, 14(11), e0224037.

Contribution: In this work I performed the laboratory analysis, specifically the taxonomic identification of the pollen collected by bumblebees through DNA metabarcoding.

2. Biella, P., Akter, A., Muñoz-Pajares, A. J., Federici, G., Galimberti, A., Jersáková, J., ... & Mangili, L. (2021). Investigating pollination strategies in disturbed habitats: the case of the narrow-endemic toadflax *Linaria tonzigii* (Plantaginaceae) on mountain screes. *Plant Ecology*, 222(4), 511-523.

Contribution: In this work I participated to the field activities and laboratory activities of DNA extraction and sequencing.

3. Scaccabarozzi, D., Guzzetti, L., Phillips, R. D., Milne, L., Tommasi, N., Cozzolino, S., & Dixon, K. W. (2020). Ecological factors driving pollination success in an orchid that mimics a range of Fabaceae. *Botanical Journal of the Linnean Society*, 194(2), 253-269.

Contribution: In this work I participated to the statistical analysis of results and revision of the manuscript.

4. Costanzo, A., Tommasi, N., Galimberti, A., Scesa, G. C., Ambrosini, R., Griggio, M., ... & Rubolini, D. (2020). Extra food provisioning reduces

extra-pair paternity in the lesser kestrel *Falco naumanni*. *Journal of Avian Biology*, 51(9).

Contribution: In this work I performed the laboratory analysis and contributed to manuscript writing.

5. De Agostini, A., Caltagirone, C., Caredda, A., Cikatelli, A., Cogoni, A., Farci, D., ... & Cortis, P. (2020). Heavy metal tolerance of orchid populations growing on abandoned mine tailings: A case study in Sardinia Island (Italy). *Ecotoxicology and Environmental Safety*, 189, 110018.

Contribution: In this work I performed the DNA barcoding laboratory analysis and participated to manuscript writing.

6. Campanaro, A., Tommasi, N., Guzzetti, L., Galimberti, A., Bruni, I., & Labra, M. (2019). DNA barcoding to promote social awareness and identity of neglected, underutilized plant species having valuable nutritional properties. *Food Research International*, 115, 1-9.

Contribution: In this work I participated to data collection, manuscript writing and revision.

7. Guzzetti, L., Fiorini, A., Panzeri, D., Tommasi, N., Grassi, F., Taskin, E., ... & Labra, M. (2020). Sustainability perspectives of *Vigna unguiculata* L. Walp. Cultivation under no tillage and water stress conditions. *Plants*, 9(1), 48.

Contribution: In this work I participated to field activities and manuscript revision.

8. Frigerio, J., Gorini, T., Galimberti, A., Bruni, I., Tommasi, N., Mezzasalma, V., & Labra, M. (2019). DNA barcoding to trace Medicinal and Aromatic Plants from the field to the food supplement. *Journal of Applied Botany and Food Quality*, 92, 33-38.

Contribution: In this work I participate to the laboratory analysis of DNA barcoding

9. Maggioni, D., Tatulli, G., Montalbetti, E., Tommasi, N., Galli, P., Labra, M., ... & Galimberti, A. (2020). From DNA barcoding to nanoparticle-based colorimetric testing: A new frontier in cephalopod authentication. *Applied Nanoscience*, 1-8.

Contribution: In this work I performed the DNA isolation and participated to manuscript writing

10. Guzzetti, L., Panzeri, D., Ulaszewska, M., Sacco, G., Forcella, M., Fusi, P., ... & Labra, M. (2021). Assessment of dietary bioactive phenolic compounds and agricultural sustainability of an African leafy vegetable *Corchorus olitorius* L. *Frontiers in Nutrition*, 8.

Contribution: In this work I participated to field activities and manuscript revision.

11. Taskin, E., Boselli, R., Fiorini, A., Misci, C., Ardeni, F., Bandini, F., ... & Puglisi, E. (2021). Combined Impact of No-Till and Cover Crops with or without Short-Term Water Stress as Revealed by Physicochemical and Microbiological Indicators. *Biology*, 10(1), 23.

Contribution: In this work I participated to field activities and manuscript revision.

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