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RESEARCH ARTICLE

Increasing road cover in urban areas is associated with greater midgut histological damage in a primitively eusocial bee

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Abstract
Urbanisation is associated with air and soil pollution, particularly from heavy metals. One of the tissues most exposed to such pollutants is the midgut epithelium as insects may ingest these pollutants with food. Bees are one of the most important urban insects, providing important ecosystem services such as pollination. However, to the best of our knowledge, no studies have investigated the possible histological alterations to the midgut epithelium of bees caused by urbanisation. We sampled workers of the ground-nesting, primitively eusocial bee *Halictus scabiosae* in a large metropolis (Milan), with the aim to test if individuals from areas characterised by higher urbanisation and consequently higher pollution levels—defined here by a greater proportion of roads—exhibit greater histological tissue and cellular alterations in the midgut epithelium. We obtained semi-thin sections of the midgut through histological techniques, and then adopted a semi-quantitative approach to assess morphological damage. The midgut presented a range of histological alterations including epithelium disorganisation, vacuolisation, and nucleus karyorrhexis (one of the stages of cellular death). We found higher histological damage score (calculated taking into account all found alterations) and frequency of karyorrhectic nuclei in sites with a higher proportion of roads (i.e. more urbanised). The observed alterations may underline a potential impairment of the digestive function in highly urbanised areas.

Keywords Histology · Midgut epithelium · Wild bees · Urbanisation · *Halictus*

Introduction

Bees (Hymenoptera: Apoidea) are the most effective insect pollinators in different environments (Lowenstein et al. 2015; Bernauer et al. 2022), playing a pivotal role in ecosystem services. Due to the ecological and economic importance of pollination (Porto et al. 2020), the scientific community is paying increasing attention to the health status of bees (De Jong and Lester 2023). This is a particularly relevant topic in disturbed landscapes, where anthropogenic pressures are driving strong environmental changes (Ohler et al. 2023). One of the most important drivers of land-use changes is the process of urbanisation, which leads to the replacement of natural areas with cemented (i.e. impervious) surfaces. This change in land cover has a significant impact on the quality of the environment occupied by bees, with a possible simplification of wild bee communities (Fortel et al. 2014) or effects at individual levels on morphological, molecular, or behavioural functional traits of these insects (Polidori et al. 2023). These effects may not necessarily be negative, as some of the shifts in functional traits may be seen as an adaptation to urban environments (Ferrari et al. 2024a, b). While the domestic honeybee is a target species for many studies on pollinators' health in disturbed habitats, wild bees were relatively less studied in such context. In particular, the consequences of their exposure to anthropogenic stress, including urbanisation, were mainly evaluated at the community-level in terms of abundance, diversity, and functional species composition (Ferrari and Polidori 2022; Braman et al. 2023; Geppert et al. 2023). To a lesser extent, wild bees in urban habitats have also been shown to be affected at the intraspecific level, with changes in external morphological traits such as body size and wing asymmetry (Banaszak-Cibicka et al. 2018; Austin et al. 2022; Ferrari et al. 2024a, b).

Urbanisation is associated not only with more cemented surfaces and fragmentation of green spaces, but also with

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increased air and soil pollution (Martin et al. 2023), the effects of which on wild bees are even less understood, with essentially all the available evidence based on honeybees. One of the most widespread pollutants in cities are heavy metals, mainly produced by industry, car exhaust and friction between mechanical metal parts such as car brake discs or train railways (Wu et al. 2022). Albeit some heavy metals—such as iron (Fe) (Nichol et al., 2002)—are important elements for organisms, most of them are highly toxic (e.g. lead (Pb) and mercury (Hg), Bosancic et al. 2020). These metals, since they do not decay, can accumulate in the soil and then be transferred to plants and phytophagous insects through biomagnification processes (Perugini et al. 2011; Sawidis et al. 2011). In addition, heavy metals can accumulate in the particulate matter (PM): a mixture of solid particles and liquid droplets found in the air that are rich carriers of heavy metals such as lead and iron (Sharma et al. 2020). Indeed, several studies used honeybees as well as honeybee products (e.g. honey) as bioindicators of air pollution (Costa et al. 2018; Di Fiore et al. 2022). For instance, honeybees tend to show higher concentrations of Pb in urban areas compared to more natural sites (Perugini et al. 2011). Ultimately, air or soil pollution might affect plant–bee interactions, with detrimental consequences on the ecosystem service of pollination (Duque and Steffan-Dewenter 2024). However, most of the studies conducted in urban environments lack a morpho-physiological approach aimed to investigate the effects of heavy metal intoxication on bees at cell and tissue level.

One of the anatomical systems most exposed to air or soil pollutants in insects is the digestive system, so studying the effects of urbanisation on its health deserves attention. In bees, the digestive system is organised in three main regions: the foregut (ingestion), the midgut (digestion and absorption), and the hindgut (absorption and excretion) (Caccia et al. 2019). The midgut, which is the only endodermal portion, is the tract most likely to be affected by anthropogenic contaminants as its epithelium represents an interface between the internal and the external environment. This is also the almost sole epithelial tissue by which nutrients are acquired. The midgut has a rounded transversal section with different layers of cells. Externally, there is a double muscle layer: inner circular and outer longitudinal muscles (Ceylan et al. 2019). The main cellular type of the epithelium is the columnar cell. They have an elongated shape and in the apical portion (i.e. towards the lumen) they present a brush border made of microvilli. The nuclei are very conspicuous and lie either in the middle or towards the base of the cell (Mitchell, 1941). These cells are responsible for the synthesis of digestive enzymes and the subsequent absorption of the nutrients (Holtof et al., 2019). In the lumen, the food mass is usually enclosed in several thin, irregularly concentric coverings of chitin and proteins embedded in a proteoglycan matrix called peritrophic membranes (Richards and Richards 1977). These membranes are gel-like structures that line the midgut, protect the cell microvilli from abrasion by food particles, and delimit the site of action of several classes of hydrolytic enzymes (Hegedus et al. 2009).

Midgut histological alterations have been used as markers of stress in honeybees, especially under laboratory conditions. However, studies about the effect of urbanisation, and hence heavy metal pollution, on the midgut epithelium of these insects are largely lacking. In laboratory experiments, Dabour et al. (2019) found that (Pb) and cadmium (Cd) oxide intoxication caused several ultrastructural changes in the cells of the midgut epithelium in honeybees, such as brush border detachment or increased cytoplasmic proteolysis. Within Hymenoptera but outside bees, Polidori et al. (2018) showed that in *Polistes dominula* (Hymenoptera: Vespidae), individuals collected at urban sites had abundant heavy metal spherites, broken and disorganised microvilli, a high amount of heterochromatin in the nuclei of epithelial cells, increased cytoplasmic vacuolisation and mitochondrial disruptions. Altogether, such morphological alterations may result in physiological impairments of the digestive functions (Zajdel et al. 2023), ultimately compromising insect fitness. Indeed, a reduced colony growth in bumblebees (Sivakoff et al. 2020; Scott et al. 2022) and an increasing proportion of dead offspring in a wild bee species (Moroń et al. 2014) were found under exposure to environmental heavy metal pollution.

Here, we aimed to evaluate for the first time the effects of urbanisation on the midgut epithelium of a wild bee, thus contributing to filling the knowledge gap both in terms of the studied habitat (lack of gut histology studies in urban environments) and of the studied model (the gut histology of wild bees has never been studied in disturbed habitats). We focused on the ground-nesting, primitively eusocial bee *Halictus scabiosae* (Rossi, 1790) (Hymenoptera: Halictidae) that was sampled along an urbanisation gradient in the Metropolitan City of Milan (Northern Italy). We hypothesised that individuals collected from urbanised areas—defined here by a greater proportion of roads and likely associated with an increased heavy metal pollution (Piscitelli et al. 2019; Bartkowiak et al. 2024)—would show increased histological alterations in the midgut epithelium.

Materials and methods

Sampling activity and landscape characterisation

The study was performed in the Metropolitan City of Milan (centre of the sampling area: 45°28′01″ N; 9°11′24″ E) and the nearest semi-natural outskirts situated in Lombardy, northern Italy. We selected a total of 10 sampling sites

(Fig. S1) along an urbanisation gradient, separated by more than 1 km to avoid possible pseudo-replication. *Halictus scabiosae* is a social, ground-nesting and polylectic bee species that forages mainly on Asteraceae and Lamiaceae (Ulrich et al. 2009). This species is very common both in urban and semi-natural areas, and easy to identify in the field.

For each site, we extracted the land-use variables from 500 m buffers, which never overlapped, created from the shapefile provided by DUSAF6.0 (https://www.dati.lombardia.it/Territorio/Dusaf-6-0-Uso-del-suolo-2018/7rae-fng6). We categorised each land-use class into the following categories: impervious areas (i.e. buildings and roads), green areas (i.e. crops, meadows, orchards, trees or urban parks) and water. For each category, we calculated the relative proportion, thus describing each sampling site in terms of relative abundance of different land-uses. Sampling sites were assigned with a specific composition of these land-use variables. Furthermore, the sites were assigned to the category "Urbanised", or "Green", based on this characterisation. Urbanised sites have (average ± standard deviation) 7.928% ± 4.284% of green areas and 18.119% ± 29.975% of road cover, while green sites have on average 70.865% ± 23.428% of green areas and 0.644% ± 1.088% of road cover (Fig. S2). We chose road cover as a possible proxy for urban air pollution levels, which are typically associated with higher levels of lead (Piscitelli et al. 2019; Bartkowiak et al. 2024), and because it well describes the difference between urban and green areas.

We sampled 50 females from 10 sampling sites (24 bees from 5 urbanised sites and 26 bees from 5 green sites) between May and July 2023, the period in which workers are on flight. The ten sampling sites were selected to create an urbanisation gradient as much representative as possible of the studied location. For instance, this gradient was created sampling the bees in urban parks, large sub-urban green areas and a low urbanised area outside the Metropolitan City of Milan (Fig. S1). Bees were hand-netted on flowers and placed in 2 cm diameter plastic tubes covered with a foam rubber lid. These tubes allow the bees to remain alive during their transport to the laboratory.

Gut extraction and microscopy

Once carried to the laboratory, bees were anesthetised for around 20 min in a fridge (4 °C). Then, they were placed on a petri dish and, with the use of forceps, the gut was gently removed pulling from the sting and immediately placed in 1.5 mL centrifuge tubes filled with fixative buffer (1:1 Cacodylate buffer 0.2 M pH 7.4 and Glutaraldehyde 4%). The guts were stored in the fixative buffer overnight at 4° C. Then, the guts were transferred from the fixative buffer to a solution with Osmium (1:1:2 Osmium 4%, dH_2O and Cacodylate buffer 0.2 M) for 2 h, keeping it away from light. The samples were then washed two times with a wash buffer (1:1 fixative buffer and dH_2O) and washed with dH_2O for two times before starting the dehydration protocol. The samples were dehydrated in a progressively increasing alcoholic series: EtOH 25% (2 times, 1 h each), EtOH 50% (2 times, 1 h each), EtOH 70% (2 times, 1 h each), EtOH 90% (2 times, 15 min each), EtOH 96% (2 times, 15 min each) and finally EtOH 100% (3 times, 20 min each). Dehydration was immediately followed by the resin embedding protocol. The protocol consisted of washing the samples in propylene oxide (4 times) followed by three different Propylene oxide–Epoxy resin mixture solutions (3:1, 1:1 and 1:3 of oxide/resin, 1 h for each step). Finally, samples were included in pure Epoxy resin at room temperature prepared as follows: Epon Araldite-812 (e.g. Fluka-Merck) four components (called A/M, B, C, and D); 54 g of component A/M, 44.5 g of component B, 10 g of component D and 2.5 g of component C. Once included, the samples were placed in an oven at 65° C for more than 48 h.

The embedded samples were cut into 0.99 µm sections with Ultramicrotome Reichert Jung Ultracut E, then stained. The staining protocol consisted in fixing the sections to microscope slides with sodium methoxide (20 s) then washing the slides in methanol (MetOH 100%) followed by ethanol (EtOH 100%), tap water and distilled water (dH2O), before colouring with crystal violet 1% w/v (1 min) and basic fuchsine stain 1% w/v (40 s). The stained sections were then covered with coverslips using Eukitt mounting medium. Microscope slides were then observed and photographed at 12.5 × with a light transmission optical microscope (Jenaval), mounted with a Leica EC3 camera (software: Leica Application Sute LAS EZ version 1.8.0).

Evaluation of midgut damage

We assessed the damage both qualitatively and quantitatively. Histological changes were first identified and described by comparison with the relevant literature (Table 1), then evaluated by one author and finally confirmed independently by two other authors, who were unaware of the origin of the samples shown. This was done to minimise bias due to possible subjectivity in the description of histological changes.

Qualitatively, we described the general appearance of the histological sections. To quantify damages, for each bee, we considered three different histological cross-sections; for each section, damages were considered in three non-overlapping regions of interest (ROIs) of 50 μm^2. To better quantify histological damages of the midgut, we followed the semi-quantitative method of *Damage score* (Grella et al. 2019). We divided the morphological damages in three score ranks: 1 (minor histological injuries),

Table 1 Description and scores attributed to each alteration recorded

Alteration	Description	Measure	Score rank
Cell elimination	Presence of fragments of epithelial cells occupying the lumen (e.g. Moreira et al., 2022)	Presence or absence	1
Vacuolisation and/or loss of cytoplasmic material	Epithelial cells present vacuoles in the apical portion (i.e. towards the lumen) (e.g. D'Urso et al. 2017)	Number of vacuoles in each ROI	2
Fragmentation or loss of brush border	The brush border is not intact and thick, or it is completely absent (e.g. Bernardes et al., 2022)	Presence or absence	2
Loss of cell contact	Epithelium appears disorganised with deformed epithelial cells and larger intercellular spaces, especially at the base of the epithelial cells (e.g. Carneiro et al., 2022)	Presence or absence	3
Karyorrhexis	Cells present nuclei with fragmented chromatin (e.g. Silva-Zacarin et al. 2008)	Relative proportion of nuclei showing karyorrhexis on total visible nuclei in each ROI	3

2 (moderate but reversible injuries), and 3 (serious and irreversible damages) (Table 1). The frequencies of the occurrence of each alteration were recorded looking at all the cells in each ROI for all the sampled bees. For quantitative metrics (e.g. number of vacuoles), we assigned a frequency of occurrence. The level of frequency was based on the median among all the sampled bees. If a sample had a higher value than the median, then a high frequency was assigned (2); otherwise, a low frequency (1), or absence of the damage (0) was assigned. For damages that cannot be counted (e.g. fragmentation of brush border), only presence (1) and absence (0) was recorded. A final *damage score* was calculated multiplying the rank of the damage by its frequency (after calculating the average between the three ROIs and three cross-sections for each bee). Image analyses were performed using the software ImageJ (Schneider et al. 2012).

Statistical analysis

We used linear mixed models to test for possible effects of the proportion of roads on the recorded histological parameters. For presence/absence data, we used a binomial distribution. All the models included the sampling site as a random effect. The analysis was performed in R Software (*v. 4.3.2*, R Core Team 2023). Models were performed with the package *glmmTMB* (Brooks et al. 2017), while normality of the data, residuals, and random effects were visually checked with the package *performance* (Lüdecke et al. 2021). Graphs were made with *ggplot2* (Wickham and Chang, 2016) and *ggbreak* (Xu et al. 2021). All the data are reported in the supplementary file Dataset.xlsx.

Results

Overall, we recognised five types of midgut alterations in the observed individuals (schematised in Fig. 1): cell elimination in the lumen, vacuolisation and/or loss of cytoplasmic material, fragmentation or loss of brush border, loss of cell contacts in the epithelium, and nuclei with fragmented chromatin (karyorrhexis). Full description of these alterations and their corresponding evaluation type and score rank can be found in Table 1.

We found an overall higher *damage score* in the urbanised sites than in the green sites (Table 2, Fig. 2A). In addition, we found a statistically significant increase in the damage score with increasing road cover in the sampling site (Table 3, Fig. 2C). In the urbanised sites, the

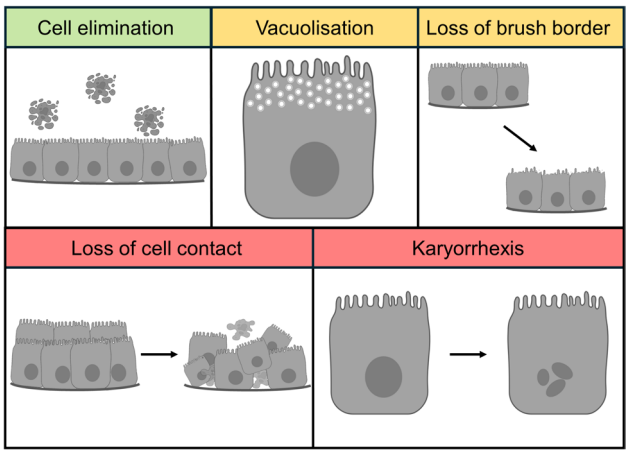

Fig. 1 Schematic representation of the histological damage to the epithelium of the midgut that we have recorded. Green: score rank of 1, Yellow: score rank of 2, Red: score rank of 3

Table 2 Summary statistics of the parameters measured to quantify damage to the midgut epithelium. For quantitative measures, mean ± standard error is given

Parameter	Urbanised	Green
Damage score	9.292 ± 0.055	8.308 ± 0.535
Vacuoles	15.549 ± 0.831	16.372 ± 0.823
Vacuoles frequency (1 = low, 2 = high)	1.458 ± 0.102	1.539 ± 0.098
Relative proportion of nuclei showing karyorrhexis	0.488 ± 0.017	0.445 ± 0.018
Karyorrhexis frequency (1 = low, 2 = high)	1.583 ± 0.101	1.423 ± 0.097
Bees with fragments of epithelial cells in the lumen	11	9
Bees with fragmented or lost brush border	10	6
Bees with loss of epithelial cell contact	2	1

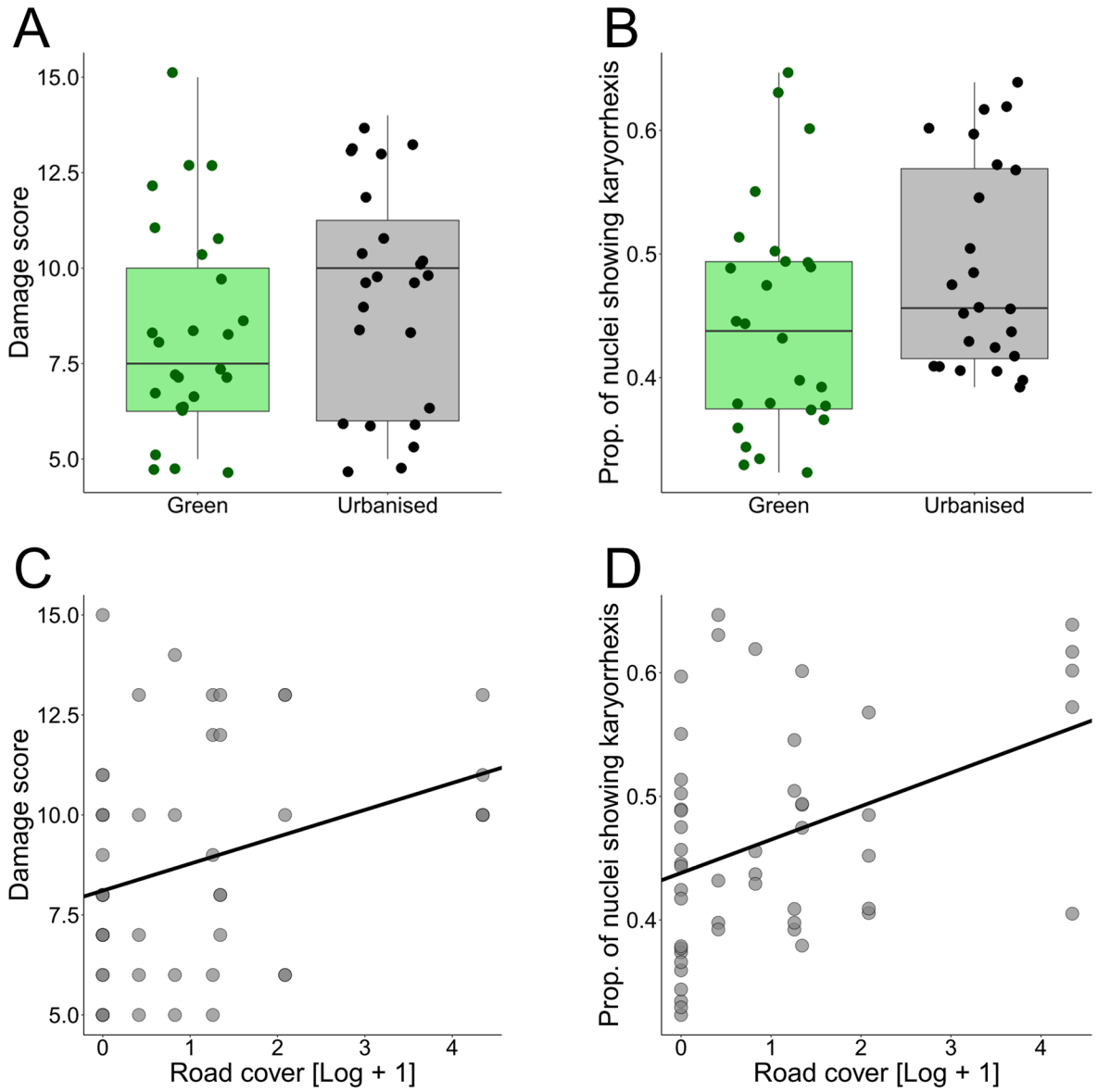

Fig. 2 Graphical representation of the statistically significant linear mixed models. In all the plots, points represent the actual value. In the scatterplots, the intercept and slope of the line are extrapolated from the model

Table 3 Linear mixed models used to test the effect of road cover (Log + 1) on the parameters measured to quantify damage to the midgut epithelium

Parameter	Term	Estimate	S.E	Z	P
Damage score	Intercept	8.108	0.520	15.597	**< 0.001**
	Road cover	0.673	0.314	2.145	**0.032**
Vacuoles	Intercept	15.249	0.733	20.801	**< 0.001**
	Road cover	0.720	0.443	1.625	0.104
Vacuoles frequency	Intercept	−0.477	0.426	−1.118	0.263
	Road cover	0.502	0.309	1.623	0.105
Proportion of nuclei showing karyorrhexis	Intercept	0.438	0.017	26.250	**< 0.001**
	Road cover	0.027	0.010	2.730	**0.006**
Karyorrhexis frequency	Intercept	−0.449	0.412	−1.090	0.276
	Road cover	0.470	0.281	1.673	0.094
Fragments of epithelial cells in the lumen	Intercept	−0.5860	0.3814	−1.536	0.124
	Road cover	0.1727	0.2225	0.776	0.438
Fragmented or lost brush border	Intercept	−1.064	0.400	−2.660	**0.008**
	Road cover	0.287	0.225	1.272	0.203

Bold values are statistically significant results ($P < 0.05$)

epithelium is often generally disorganised, often with massive release of cells in the lumen (Fig. 3A–C and 4B), or with intense vacuolisation in the medial-basal portion of the epithelium (Fig. 3B and 4D). In fact, we found 11/24 bees in the urbanised areas and 9/26 bees in the green areas with a presence of epithelial cell fragments in the lumen (Table 2), although this parameter did not change statistically significantly with road cover (Table 3). Conversely, in green areas, the epithelium shows greater uniformity between cells (Fig. 3D-F, Fig. S3) and we could not find a massive release of cells in the lumen (Fig. 3D–F, Fig. S3).

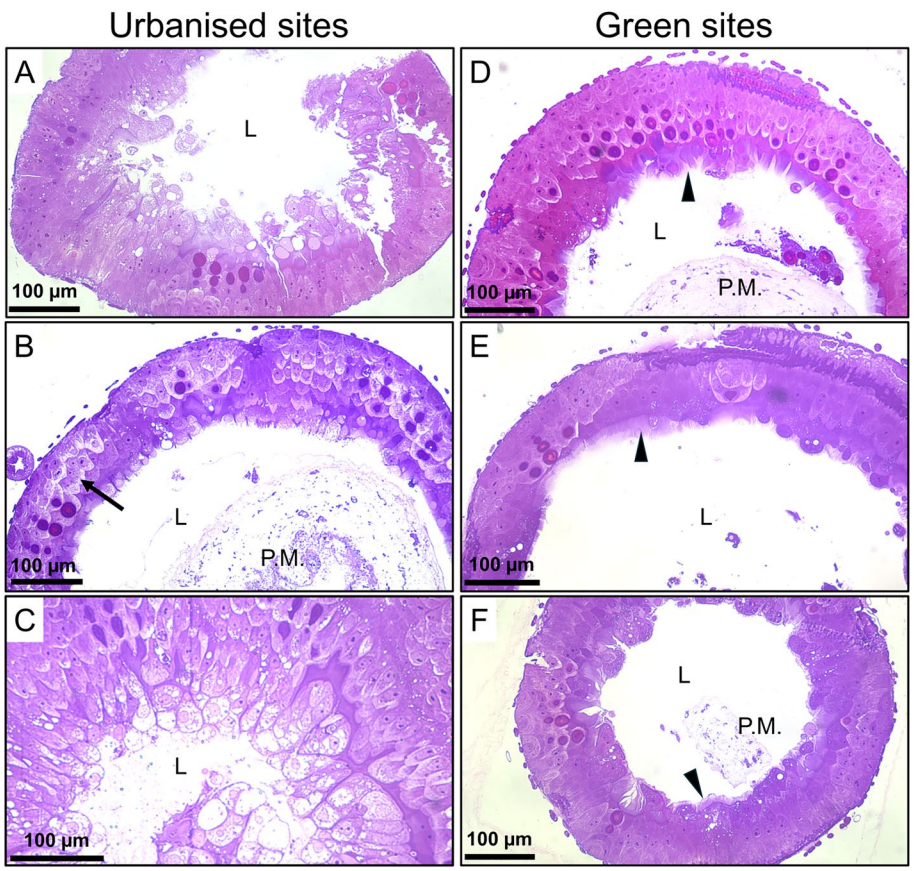

Fig. 3 Histological sections of the midgut epithelium from three bees collected from the urbanised sites **(A–C)** and three bees from the green sites **(D–F)**. Sections **A** and **C** show an epithelium with massive release of cells in the lumen (L). Section B shows an epithelium with massive vacuolisation (black arrow) in its medio-basal part. Sections **D, E,** and **F** show a compact epithelium with the brush border clearly visible (arrowheads) and without extensive vacuolisation or release of cell in the lumen (L). *P.M.* peritrophic membrane

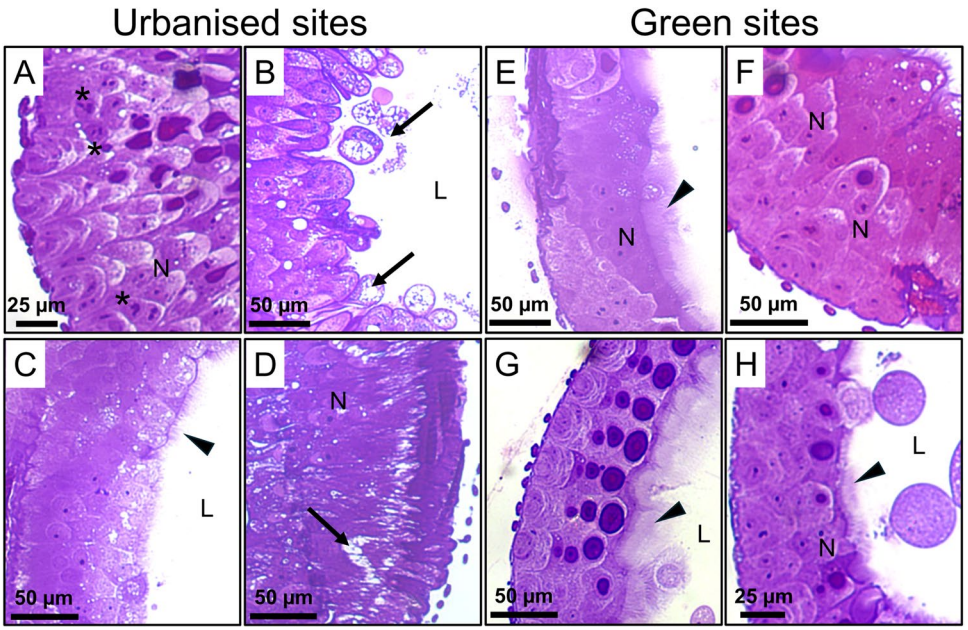

Fig. 4 Higher magnification of histological sections of the midgut epithelium from four bees collected from the urbanised sites **(A–D)** and four bees from the green sites **(E–H)**. Section **A** shows an epithelium with a high frequency of karyorrhectic nuclei (asterisks) compared to normal nuclei (N). **B** shows a magnified view of cells with vacuoles released in the lumen (L). Section **C** shows a thin and damaged bush border (arrowhead), while section **D** shows an extensive vacuolisation in the basal part of the epithelium. **A** thick and intact bush border (arrowheads) can be seen in sections **(E, G, and H**; while a large presence of normal nuclei (N) c) an be seen in sections **(E, F, and H)**

The higher damage score was reflected in the higher frequency of certain damages that we recorded. For example, the relative proportion of nuclei showing karyorrhexis was on average higher in urbanised areas (1.583) then in green areas (1.423) (Table 2, Fig. 2B) and statistically significantly higher in sites with a higher proportion of road cover (Table 3, Fig. 2D). Karyorrhexis is a form of cell degeneration, which involves the fragmentation of the cell nucleus into several parts of different sizes. These nuclei often have more than 3 dense chromatin fragments (Fig. 4A) and can be distinguished from other nuclei that have only one heavily stained portion (Fig. 4E–H). In addition, we found 10/24 bees in urbanised areas and 6/26 bees in green areas with a fragmented or complete loss of brush border in urban areas (Table 2, Fig. 4C–D). In fact, bees from green sites often had a smooth and thick brush border, clearly visible in the apical part of the epithelial cells (Fig. 4G–H, Fig. S3). However, this parameter did not change statistically significantly with road cover (Table 3). Complete disintegration of the epithelium was found in only 3/50 bees (Table 2) and was, therefore, not analysed or discussed further.

Discussion

Here, we investigated for the first time the effects of urbanisation on histological changes in the midgut epithelium of the wild bee species *H. scabiosae*, highlighting a novel aspect of how cities affect this widely spreading urban-dweller pollinator (Gil-Tapetado et al. 2024). Previous studies have shown that metals, including heavy metals, cause severe histological and cytological damage in bees under laboratory stress conditions. Confined to the midgut, both the epithelium and the peritrophic membrane of honeybees (Dabour et al., 2019) and stingless bees (Bernardes et al. 2022) showed alterations due to metal exposure. Other tissue and cell damage, ranging from the hepato-nephrocyte system to haemocytes and oenocytes, was also observed in metal-exposed honeybees and bumblebees (Camargo Abdalla and Costa Domingues, 2015; Polykretis et al. 2016; Nogueira et al. 2019; Caliani et al. 2021).

The semi-quantitative approach used (Grella et al. 2019) allowed us to quantify epithelial histological damage in more detail. Indeed, we found that bees sampled from sites with increasing road cover—positively correlated with the urbanisation gradient—had increasing *damage scores*. This score takes into account all the damages we recorded (presence of cells in the lumen, vacuolisation, loss of brush border, loss of epithelial cell contact, and nuclei showing karyorrhexis) and combines their frequency by their severity score rank. This means that bees in more urbanised areas could have an overall reduced health status of the midgut epithelium. This confirms our hypothesis that urbanisation may cause histological changes in the midgut epithelium. In particular, we used the proportion of roads around the sampling site as a proxy for the level of pollution. In fact, greater road coverage is associated with an increased number of cars and probably air pollution (Hien et al. 2020). In particular, car exhaust and disc brake particles are a major source of heavy metals in urban areas (Wu et al. 2022). Bees are known to be natural samplers of heavy metal pollution, especially in urban landscapes (Perugini et al. 2011) where they suffer adverse effects from air pollution (Thimmegowda et al. 2020). Therefore, we can hypothesise that the increased damage

score we observed is probably due to increased levels of heavy metals in the air and/or in the soil.

We hypothesise that the overall increase in the *damage score* in more urbanised areas is probably due to a higher proportion of nuclei showing karyorrhexis: the histological parameter which differed between urban and green areas the most. Indeed, karyorrhectic nuclei have the highest severity rank score. Karyorrhexis is the destructive fragmentation of the nucleus of a dying cell, with chromatin irregularly distributed throughout the cytoplasm; it is usually preceded by pyknosis (Ghanim et al. 2016). This is a prelude to cell death and is common in insects suffering from tissue degeneration. Once the dying process has begun, it becomes irreversible (Silva-Zacarin et al. 2008). The suggestion that this type of cellular damage may also be associated with heavy metal pollution in areas with many roads is somehow confirmed by what was observed by Dabour et al. (2019) and Bernardes et al. (2022), who found a higher proportion of autophagic and apoptotic cells (i.e. also cells that die) in the midgut of metal-treated honeybees and stingless bees.

Finally, we found no statistically significant variation in vacuolisation, loss of brush border, or release of cells in the lumen along the road cover (urban) gradient. Although we expected an increase in these damages with increasing urbanisation, these results are not surprising given the high heterogeneity of histological damages found in the literature in the midgut of Hymenoptera exposed to pollutants (Dabour et al. 2019; Polidori et al. 2018; Skaldina et al. 2023). As some of these changes are physiological, such as the release of cells into the lumen or vacuolisation, we argue that the level of pollution to which these bees were exposed was not high enough to reveal changes in such physiological processes. In addition, we can also argue that damage to the brush border—which we assessed as a presence/absence response—may not be sensitive enough to show variation along the road cover (urban) gradient. This is somewhat supported by the fact that more precise and quantitative measures—the damage score and the proportion of nuclei showing karyorrhexis—showed significant variation along the road cover (urban) gradient.

While no previous studies on urban bee guts are available for comparisons, our results found accordance with what was observed in a couple of studies carried out on social wasps. Indeed, it was found a degenerated brush border in wasps sampled from polluted sites, as well as increased cytoplasmic vacuolisation (Skaldina et al. 2023) and condensed chromatin in pyknotic nuclei (Polidori et al. 2018; Skaldina et al. 2023). Although these authors only used a qualitative assessment of the damages, their results are largely consistent with our findings. However, we did not find traces of spherites or encapsulated granules of heavy metals in the vacuoles of epithelial cells, which have been found in social wasps (Polidori et al. 2018; Badejo et al. 2021). This was quite surprising, given that sequestering metals as mineralised spherites into the midgut is a common detoxification mechanism in insects, including bees (Polykretis et al. 2016). One hypothesis is that in *H. scabiosae* these spherites are deposited in other structures here not studied, for example the Malpighian tubules (Polidori et al. 2018).

Another possibility is that the heavy metal pollution level in our study area (urban and semi-natural parks) is lower than that present in previously studied areas. This is probable for the study of Badejo et al. (2021) which was conducted in a smelter. However, Polidori et al. (2018) found spherites in a study carried out in Valencia (Spain), which we argue can present comparable levels of air pollution, given that in both these cities the heavy metal pollution has been documented (Contardo et al. 2020; Martín et al. 2015). Honeybees have been largely used as natural bioindicator of heavy metal pollution in urban areas often documenting high levels of contamination (Costa et al., 2018, 2021). However, since these types of studies process the whole body of the bee, we argue that the high values of heavy metal contamination found comes from the methodology applied—whole body versus only the midgut in our case. Nevertheless, the reason why we could not find any spherites remain to be further investigated as we still know very little about such detoxification mechanisms (Borsuk et al. 2021).

Taken together, these studies show that urbanisation can indeed cause damage to the digestive system of Hymenoptera, an aspect that has been largely neglected while studying the effects of this wide anthropogenic disturbance on the morpho-physiological traits of bees. In addition, the digestive system of bees also hosts a rich microbiota. Indeed, airborne pollution containing heavy metals may derive from several traffic-related processes such as brake abrasion or combustion processes that are known to alter the microbiome of bumblebees (Seidenath et al. 2023). These changes are related to metabolism and signal transduction, which indicates a general stress response. This would add another layer of investigation for future studies.

Nevertheless, our study has some non-negligible shortcomings as we were only interested in the histological aspects. For instance, our method cannot infer possible functional changes in the midgut epithelium, which is capable of producing digestive enzymes and supporting vectorial transport of small organic nutrients, ions, and water (Caccia et al. 2019). Histological damage to the midgut epithelium may also have been caused by the presence of pathogens known to infect *Halictus* species (Cilia et al. 2022, 2023). For example, *Nosema ceranae* has been widely reported to cause epithelial damage to the midgut of bees (Dussaubat et al. 2012; Gisder et al., 2020). Although we cannot completely exclude this possibility, we did not detect *N. ceranae* or a massive spore production in midgut epithelial cells typical of these

infections. Therefore, the damages detected in our samples may be more probably due to urbanisation.

In conclusion, we presented the first evidence of midgut epithelial damage in a wild bee species in an urban environmental context. Although we did not perform a functional analysis, we can speculate that a damaged epithelium would lead to an impairment of digestive functions with possible consequences for bee fitness (Hu et al. 2019), although future studies are certainly needed to really test these hypotheses, for example by analysing midgut enzymatic activity in detail. In addition, further studies should also measure the heavy metal content of the bees collected along an urbanisation gradient and test for correlation between such variable and midgut histological or physiological alterations. Finally, further studies on other non-*Apis* species, both solitary and eusocial, would also increase our knowledge about this topic and give insights on possible differential responses by different taxa or related with different life-histories.

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Author contributions CP, AF, and FB conceived the study. CFT and AF collected the data. CP, AF, and FB analysed the data. AF wrote the first draft of the manuscript, with subsequent contributions from CP and FB. All the authors read, improved, and approved the manuscript.

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Data availability All the data are contained in the file Dataset.xlsx.

Declarations

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

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