Article

NEW ECOLOGICAL INSIGHTS ON WILD POLLINATOR *ANDRENA HESPERIA*

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Journal of Pollination Ecology, 37(19), 2024, 303-325
DOI: 10.26786/1920-7603(2024)796

Received 17 April 2024, accepted 22 November 2024

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Abstract—A nesting aggregation of *Andrena hesperia* in Bologna (Italy) was studied to characterize this bee's ecology, phenology and interactions with the environment. *Andrena hesperia* adults emerged between the end of March and the middle of April, displaying univoltine, protandrous phenology. The average resistance to heat stupor of *A. hesperia* females was 42.53 ± 13.77 minutes at 40°C. Parasites *Nomada facilis* and *Bombylius canescens* were associated with the nests. DNA barcode sequences (COI gene) of *A. hesperia* and *N. facilis* were sequenced and deposited in GenBank. The gut microbiota of newly emerged individuals was dominated by Bacillota (*Lactobacillus* and *Fructobacillus*) and Pseudomonadota (*Snodgrassella alvei* and *Gilliamella*). The pollen carried by *A. hesperia* females was identified morphologically as belonging for the most part to the Asteraceae family. The pathogens detected on *A. hesperia* showed different infection loads in newly-emerged individuals and foraging adults. This is the first time that comprehensive information on *A. hesperia* is reported, and it will hopefully foster further studies on this wild bee.

Keywords—*Chrysandrena*; pollen spectrum; microbiota; brood parasite; interspecies transmission; heat stupor

INTRODUCTION

Pollination is a key process in natural ecosystems, since almost 90% of flowering plant species benefit from zoogamic pollination (Ollerton et al. 2011). From a human perspective, pollinators contribute to about 35% of global crop production by volume (Klein et al. 2007). Among pollinators, bees perform more than half the total number of flower visits (Rader et al. 2016). They therefore play a pivotal role in plant pollination, since a massive number of plant visits is necessary to collect pollen to feed their offspring (Müller et al. 2006). In recent years, policymakers and the general public have expressed concern about the conservation status of pollinators, including wild bees, in the face of increasing risk (Althaus et al. 2021). This attention has fostered intergovernmental protection initiatives and renewed monitoring, like those promoted by the European Union (Potts et al. 2020).

Wild bees play a fundamental role in the pollination of wild and crop plants but are threatened by a variety of abiotic and biotic factors. These factors include pesticides (O'Neal et al. 2018; Siviter et al. 2018; Hrynko et al. 2021), habitat fragmentation (Hung et al. 2021; Librán-Embid et al. 2021), climate change (Schenk et al. 2018; Duchenne et al. 2020; Cane 2021), urbanisation (Fortel 2014; Choate et al. 2018; Hofmann & Renner 2020; Wenzel et al. 2020), competition with managed bees (*Apis mellifera* and *Bombus* spp.) (Rhodes 2018; Rasmussen et al. 2021), pathogens, parasites (Nanetti et al. 2021a) and introduced alien species (Vanbergen et al. 2017). The updated checklist of European bees counts a total of 2138 species of wild bees belonging to 77 genera in six families (Andrenidae, Apidae, Colletidae,

Halictidae, Megachilidae and Melittidae) (Ghisbain et al. 2023). However, in the latest IUCN European red list of bees, ~4% are threatened (178 out of 1942) and for ~55% of species (1101 out of 1942) data were deficient (Nieto et al. 2014). Considering the Andrenidae family and *Andrena* genus only, the percentage of data deficient reaches 73% (Nieto et al. 2014).

Andrena Fabricius, 1775 is known to be the largest bee genus in Europe, including ~480 valid species with ~220 in Italy (Ghisbain et al. 2023; Reverté et al. 2023). *Andrena* is highly diverse and includes about 55 subgenera in Europe, although their phylogenetic value was recently challenged. Within subgenera, *Chrysandrena* Hedicke, 1933 is a polyphyletic subgenus, the species of which belong to two separate clades (Pisanty et al. 2022). Excluding the undescribed and relatively basal group incorrectly classified in *Chrysandrena*, the subgenus has typical Palearctic distribution, ranging from northern Asia to western Europe and North Africa (Pisanty et al. 2022; Bossert et al. 2022). In Italy, this subgenus is represented by *Andrena fulvago* (Christ, 1791) and *Andrena hesperia* Smith, 1853 (Reverté et al. 2023). Like all *Andrena,* these are solitary ground-nesting bees. *Andrena fulvago* and *A. hesperia* are specialized in collecting the pollen of Asteraceae (Dermane et al. 2021); and pseudocopulation by males of *A. hesperia* has been observed on *Ophrys corsica* flowers (Schatz et al. 2021). Bees of the subgenus *Chrysandrena* are known to host brood parasitic bees. Unfortunately, there is less information in this regard for *A. hesperia* than for congeneric species. To the best of the authors' knowledge, four brood parasites are reported to be associated with *A. fulvago: Nomada fabriciana* (Linnaeus 1767), *N. femoralis* Morawitz, 1869, *N. ferruginata* (Linnaeus 1767) by Comba (2019), and *Nomada facilis* Schwarz, 1967 by Notton & Norman (2017). The previous associations between species should be approached with caution, as the available information is scarce and conflicting. Unfortunately, no further information is available about the brood parasites of *A. hesperia.*

In general, pathogens of *Andrena* species are known mainly due to potential interspecies transmission with honey bees, which could play an ecological role in *Andrena* fitness (Nanetti, Bortolotti, et al. 2021). Deformed Wing Virus (DWV) is a commonly detected bee virus infecting *Andrena* species, while Acute Bee Paralysis Virus (ABPV), Chronic Bee Paralysis Virus (CBPV), Lake Sinai Virus (LSV), Black Queen Cell Virus (BQCV), Sac Brood Virus (SBV) and *Nosema ceranae* have also been detected (Singh et al. 2010; Evison et al. 2012; Ravoet et al. 2014; Dolezal et al. 2016; Melathopoulos et al. 2017; Radzevičiūtė et al. 2017; Murray et al. 2019; Cilia et al. 2022b). Bee virus variants and the new Andrena-Associated Bee Virus-1 have also been identified in some mining bees (*A. combusta, A. aerinifrons, A. urfanella* and an unidentified morphospecies of the subgenus *Truncandrena*) (Daughenbaugh et al. 2021).

The microbiota of *Andrena* species is less characterized and poorly known. *Apilactobacillus, Spiroplasma* and *Xenorhabdus* genus have been identified from *A. rudbeckiae* in the U.S.A. (Holley et al. 2022). A more complex gut microbiome has been identified in Belgian *A. vaga* (Hettiarachchi et al. 2023).

In addition to pathogens, an additional pressure on bee populations is anthropogenic stress. Pesticides and urbanization have had a strong negative impact on biodiversity in recent decades (Silva et al. 2020; Llodrà-Llabrés & Cariñanos 2022). However, urban parks, private gardens and the like can still serve as a refuge for pollinator communities (Hall et al. 2017; Silva et al. 2023). It is therefore of great interest to study the ecology of wild bees under conditions of high anthropogenic impact.

In sum, our understanding of the biology and ecology of *Andrena* bees, like for most wild bee species, remains scanty and fragmented. To conserve pollinator species, it is first essential to understand their biology, ecology, interactions with plants and potential threats. Here we aimed to collect new information on the ecology and biology of *Andrena hesperia*, observing a nesting population in a city centre in the north of Italy. To better understand how this species interacts with the environment, we gathered data on emergence timing, bee pathogen occurrence, brood parasites, parasitoids, pollen preferences, gut microbiota and heat stress.

MATERIALS AND METHODS

STUDY AREA

The municipality of Bologna, a major northern Italian city, is on the south-eastern side of the Po Plain at the foot of the Pre-Apennine hills. The area is characterized by a warm-temperate climate (Peel et al. 2007; Beck et al. 2018) with an average temperature of 14.3°C and an average yearly precipitation of 825 mm (Climate data for cities worldwide; https://en.climate-data.org), and is part of the European continental biogeographical region (Cervellini et al. 2020).

The study was conducted at the CREA-AA Research Centre, located in a discontinuously urbanised area about 3 km north of the city centre (Fig. 1). The Research Centre comprises various buildings and experimental greenhouses surrounded by meadows. There are some experimental apiaries within the Research Centre perimeter. The opportunity for the study was provided by a nesting aggregation of *Andrena hesperia*, already known from previous years, in the courtyard. The nests were distributed over an area of about 4 m^2 in one of the lawns.

MONITORING

Two kinds of monitoring were conducted on the population of *A. hesperia*: monitoring with emergence cages and net sampling on flowers.

Emergence cages were used to monitor the timing of bee emergence, to sample brood parasites and parasitoids and to collect newly emerged specimens in order to study their pathogens and microbiota. Emergence cages consisted of a wooden framework measuring 60 cm x 40 cm x 40 cm with fine netting on five faces, and an open face on the long side. The cages were positioned with the open face to the ground, closing any gaps between framework and ground with soil. Three emergence cages were set in place in the area where most of the bee nests were expected to be, before bees started appearing, based on the activity period of the species observed the previous year. The cages remained in place from 25 March to 25 April 2023 and were checked for new bees, brood parasites and parasitoids once a day after 12 midday. All newly emerged *A. hesperia* individuals were recorded daily and their sex noted. The sex ratio was calculated as males to total individuals (sex

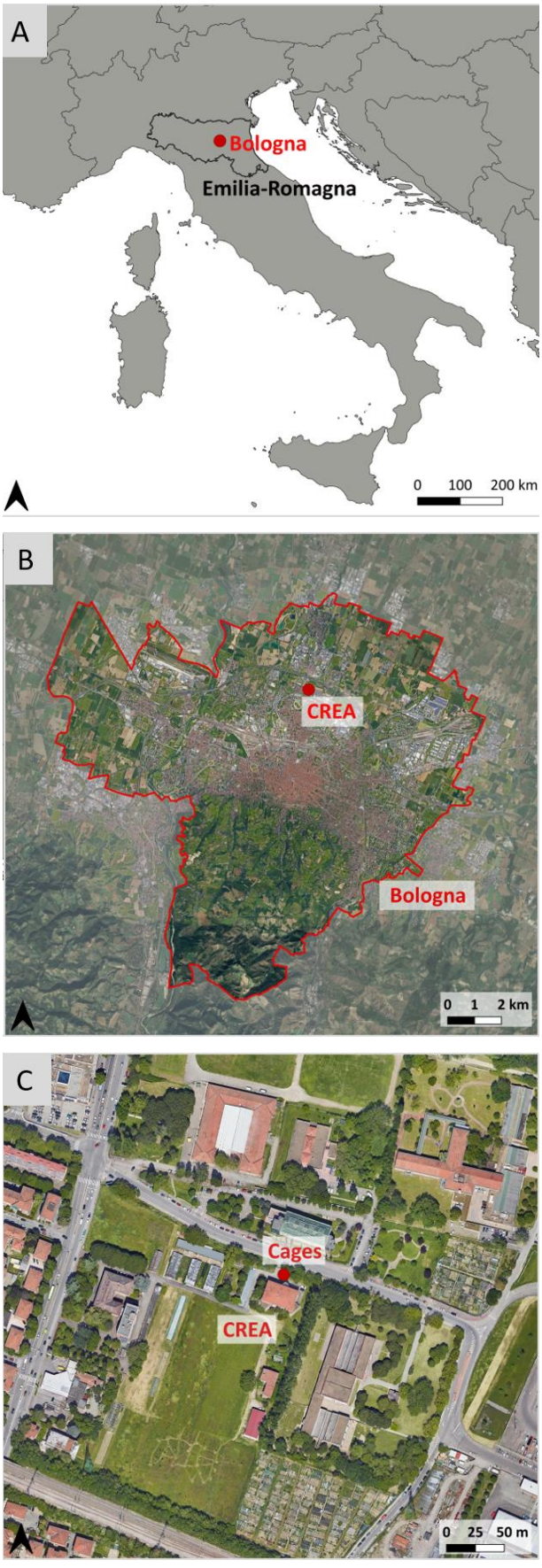

Figure 1. Study area in the city of Bologna (Emilia-Romagna, Italy).

ratio=males/(males+females)) with 95% confidence interval (C.I.).

Sampling was carried out in the meadow surrounding the nests using entomological hand nets. Bees were collected when feeding and foraging on flowers. Male and female *A. hesperia* were collected for pathogen and microbiota detection, while only females were collected for heat stress tests and pollen analysis. Net sampling continued throughout the flight period.

Taxonomic identification

In the absence of identification tools for Italian fauna, *Andrena* specimens were determined morphologically with the help of identification keys for central Europe and for the Iberian Peninsula (Wood 2023). *Andrena hesperia* Smith, 1853 is a medium-sized bee (♀ 9-10 mm, ♂ 8-9 mm), with a predominantly dark integument. Females are recognised by their shagreened and densely punctate clypeus, mesonotum and scutellum, by short foveae dorsally occupying no more than half the space between the compound eyes and the lateral ocelli, by orange mid and hind tibiae and tarsi, and by orange hairs on tergite marginal bands and hind legs. Males can be identified by red-brown tarsi, dull clypeus and mesonotum and by tergites with a narrow marginal band of yellow hairs (Schmid-Egger & Scheuchl 1997; Amiet et al. 2010; Wood 2023). *Andrena hesperia* was assigned to the subgenus *Chrysandrena* Hedicke, 1933 accordingly to the subgeneric classification derived from the latest phylogenetic review of the genus *Andrena* (Pisanty et al. 2022).

Likewise, parasitic bees of the genus *Nomada* Scopoli, 1770 were assigned to their species according to the latest phylogenetic data (Straka et al. 2024) using a morphological identification key (Smit 2018). All other insects collected in the cages were pinned and sent to specialists for identification.

DNA Barcode

Two methods were used to generate DNA barcodes. Total DNA was extracted from the right hind leg (Villalta et al. 2021), dissected from three female and three male individuals. At the same time, a sterile microbiological swab soaked with digestion buffer was rubbed gently over the sternites of each specimen of *A. hesperia*, using a specific protocol (Flaminio et al. 2023a). Legs and swabs were placed in a microtube containing 1 mL digestion buffer and incubated for 18 hours at 56°C. For the *Nomada* specimen, DNA was extracted from the hind leg after dissection. Total DNA purification was performed by phenol:chloroform extraction (UltrapureTM Phenol:Chloroform:Isoamyl Alcohol, Thermo Fisher Scientific, Waltham, MA, USA), as reported (Cilia et al. 2022a). The DNA obtained was quantified using an Infinite 200 PRO NanoQuant™ spectrophotometer (TECAN Life Technologies, Männedorf, Switzerland) and stored at -20°C until analysis. Double-distilled Rnase-Dnase-free water was used as negative control for all processes. Amplification of mitochondrial DNA (*mt*DNA) was performed using primer pairs able to amplify a 710-bp fragment in the highly-conserved region coding for the gene Cytochrome C oxidase subunit I (COI): LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HC02198 (5′-TAAACTTCAGGGTGACCAAAA AATCA-3) (Folmer et al. 1994). PCR was performed in a reaction volume of 25 µl using HotStarTaq Polymerase (Qiagen, Hilden, Germany) as previously reported (Flaminio et al. 2023b). All amplicons were visualized on 1.5% agarose gel. The amplicons were purified using ExoSAP-IT Express (ThermoFisher Scientific) and sequenced by SeqStudio™ (ThermoFisher Scientific) using standard Sanger methodology. The sequences were analysed using BioEdit (Hall 1999) to create the consensus sequence by aligning forward and reverse sequences and BLAST (using the megablast algorithm) (Altschul et al. 1990). Phylogenetic analysis with other deposited *A. hesperia* COI sequences was performed using the Maximum Likelihood method and Tamura-Nei model in MEGA X (Kumar et al. 2018).

Heat stress test

The resistance of *A. hesperia* to heat waves was evaluated as Time to Heat Stupor (THS), following the protocol developed by Martinet et al. (2015). Briefly, 39 *Andrena* females were placed in 40 ml tubes, perforated to enable ventilation, and kept at 8-12°C overnight, to acclimatize and standardize their status. The next day, they were fed ad libitum with a sugar syrup (60% sugar, 40% water). The tubes containing the bees were then placed in a Herp Nursery II (Lucky-Reptile, Waldkirch, Germany) incubator pre-heated to 40°C. The

temperature was maintained at 40°C and the bees were monitored and timed with a chronometer for signs of heat stupor, namely: i) drastic reduction or complete lack of movement; ii) falling on their backs; iii) loss of muscle coordination. Heat stupor is judged to occur when the specimen is unable to return to its normal position and shows spasms (Martinet et al. 2015). For each bee, THS was calculated as the time from insertion in the incubator to the occurrence of heat stupor.

PATHOGEN DETECTION

To assess possible interspecies transmission of common pathogens between *Apis mellifera* and *A. hesperia,* 29 newly emerged and 30 free-ranging *Andrena* specimens were analysed. All specimens were washed in 95% ethanol before extraction to eliminate any external microbiological contamination. Each bee was examined individually. The samples were homogenized using a TissueLyser II (Qiagen, Hilden, Germany), as previously reported (Cilia et al. 2019; 2022b). The DNA and RNA extractions were performed using a Quick DNA Microprep Plus Kit (Zymo Research) and a Quick RNA Microprep Plus Kit (Zymo Research), respectively, as previously reported (Mazzei et al. 2019; Nanetti et al. 2021b; Cilia et al. 2022c). The extracted nucleic acids were eluted in 200 µl DNAase-RNase-free water and kept at -80°C until qPCR analysis.

Quantitative Real-Time PCR (qPCR) analysis of the extracted DNA and RNA was performed to determine the abundance of the various pathogens in the samples. *Ascosphaera apis*, *Nosema ceranae* and trypanosomatids were detected by DNA, while viruses were investigated using RNA. The primers used for the qPCRs are reported in Table 1.

A total reaction volume of 10 µl was produced for each target gene using SYBR™ green assays with forward and reverse primers and nucleic acid extract, as reported in previous studies (Cilia et al. 2021b; 2022b). The SYBR PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific) and the Power SYBR™ Green Cells-to-CT™ Kit (ThermoFisher Scientific) were used for the DNA and RNA, respectively. The qPCRs were carried out using a QuantStudio™ 3 Real-Time PCR System (ThermoFisher Scientific), according to the protocols for each gene sequence (James &

Table 1. List of primers used to detect fungi, microsporidia, trypanosomatids and viruses.

Target	Primer name	Sequence (5'-3')	Temperature of annealing	Reference
Ascosphaera apis	A_apis_3-F1 A_apis_3-R1	TGTCTGTGCGGCTAGGTG CCACTAGAAGTAAATGATGGTTAGA	62°C	(James & Skinner 2005)
Nosema ceranae	Hsp70_F Hsp70_R	GGGATTACAAGTGCTTAGAGTGATT TGTCAAGCCCATAAGCAAGTG	63°C	(Cilia et al. 2018)
Lotmaria passim	Lp2F 459 Lp2R 459	AGGGATATTTAAACCCATCGAA ACCACAAGAGTACGGAATGC	60°C	(Arismendi et al. 2016)
Crithidia mellificae	Cmel_Cyt_b_F Cmel_Cyt_b_R	TAAATTCACTACCTCAAATTCAATAACATAATCAT ATTTATTGTTGTAATCGGTTTTATTGGATATGT	60°C	(Xu et al. 2018)
Crithidia bombi	C.bombi_119Fw C.bombi_119Rv	CCAACGGTGAGCCGCATTCAGT CGCGTGTCGCCCAGAACATTGA	64.5°C	(Huang et al. 2015)
DWV	DWV Fw 8450 DWV Rev 8953	TGGCATGCCTTGTTCACCGT CGTGCAGCTCGATAGGATGCCA	60°C	(Mazzei et al. 2018)
BQCV	BQCV 9195F BQCV 8265R	GGTGCGGGAGATGATATGGA GCCGTCTGAGATGCATGAATAC	60°C	(Chantawannakul et al. 2006)
CBPV	CPV 304F 79 CPV 371R	TCTGGCTCTGTCTTCGCAAA GATACCGTCGTCACCCTCATG	60°C	(Chantawannakul et al. 2006)
ABPV	APV 95F APV 159R	TCCTATATCGACGACGAAAGACAA GCGCTTTAATTCCATCCAATTGA	60°C	(Chantawannakul et al. 2006)
KBV	KBV 83F KBV 161R	ACCAGGAAGTATTCCCATGGTAAG TGGAGCTATGGTTCCGTTCAG	60°C	(Chantawannakul et al. 2006)
IAPV	IAPV B4S0427_R130M IAPV B4S0427_L17M	RCRTCAGTCGTCTTCCAGGT CGAACTTGGTGACTTGARGG	62°C	(Kajobe et al. 2010)
AmFV	AmFV2-F AmFV2-R	ACCCAACCTTTTGCGAAGCGTT ATGGGGCGTCTCGGGTAACCA	56°C	(Hartmann et al. 2015)

Skinner 2005; Chantawannakul et al. 2006; Kajobe et al. 2010; Hartmann et al. 2015; Huang et al. 2015; Arismendi et al. 2016; Mazzei et al. 2018; Xu et al. 2018; Cilia et al. 2018). DNA and RNA previously extracted from positive honey bees were employed as positive controls for each pathogen investigated; sterile water was used as negative control. All analyses were carried out in duplicate. A standard curve from 1×10^1 to 1×10^9 copies was created for each target gene, as previously reported (Cilia et al. 2022b; 2023a), using the amplification and quantification protocols (Arismendi et al. 2016; Chantawannakul et al. 2006; Cilia et al. 2018; Hartmann et al. 2015; Huang et al. 2015; James & Skinner, 2005; Kajobe et al. 2010; Mazzei et al. 2018; Xu et al. 2018).

Gut microbiota identification

Twenty newly emerged *A. hesperia* specimens (10 male and 10 female) were employed to investigate gut microbiota. The ventriculus (small intestine and midgut) was dissected from each bee and homogenized with 1.5 ml sterile physiological solution (Cilia et al. 2020; 2021a). The pool was ground with a microbiological micro-pestle and streaked onto different media: Tryptone Soy Agar (TSA) (Oxoid, Basington, UK) spiked with 10% defibrinated sheep blood to isolate Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria; De Man, Rogosa and Sharpe (MRS) agar (Oxoid) to isolate Lactobacillales; UTIC agar medium (Condalab, Madrid, Spain) to isolate the other bacterial strains (Cilia et al. 2023b). All plates were incubated aerobically at 37±1°C for 24h. After incubation, bacterial growth was evaluated, and the different colonies were isolated pure onto TSA.

For the identification of isolated strains, each bacterial colony was subcultured in TSA. Total DNA was extracted from colonies using Quick DNA Microprep Plus Kit (Zymo Research, Irvine, CA, USA). DNA amplification of the *16S rRNA* gene was performed with universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACT-3′) (Heuer et al. 1997). PCR was performed using HotStarTaq Polymerase (Qiagen), according to a previously reported thermal protocol (Dos Santos et al. 2019). All amplicons were visualised on a 1.5% agarose gel, purified using ExoSAP-IT Express (ThermoFisher Scientific) and sequenced by SeqStudio™ (ThermoFisher Scientific). The sequences obtained were analysed using BioEdit (Hall 1999) and bacterial strains were identified using BLAST (Altschul et al. 1990). Identification of bacterial strains was considered only if Percentage of Identity was over 96% with sequences deposited in GenBank.

Pollen identification

Pollen samples were collected from 11 specimens of *A. hesperia* captured in close proximity to the nests during the study period in spring 2023, and from an additional seven specimens conserved in the CREA-AA entomological collection, captured in different Italian regions (five from Piedmont, one from Emilia-Romagna and one from Campania) in the framework of the BeeNet Project (Giovanetti & Bortolotti 2021). Dry specimens were analysed to obtain more information about the foraging behaviour (lecty) of *A. hesperia* over a more extended area.

The specimens captured close to nests were all females that had just visited flowers, thus with pollen-loaded scopae. Pollen grains were extracted by washing specimens with 1% SDS solution (sodium dodecyl sulphate) in a TissueLyser (Lucas et al. 2018). After being pelleted, the pollen was diluted in 30 microliters of distilled water and vortexed. Two replicates of each pollen extract were prepared by placing two separate 5 microlitre drops on a slide and sealed with glycerinated gelatine after gentle drying to remove excess water.

The dried specimens of *A. hesperia* from the collection were treated differently: under the stereo microscope, the pollen was removed from the leg brushes and propodeal corbicula using a pin and dropped into a microtube placed under the specimen (Westrich 1986; Wood & Roberts 2017).

Specimens were not rehydrated to avoid over-handling and damaging them and only pollen collected from the brushes was extracted, as pollen from the rest of the body may have been picked up during nectar collection, as previously reported (Westrich et al. 2015). In order to compare methods, the pollen was diluted in the same volume and the preparation of slides was the same as for the previous samples.

Morphological identification and counting of pollen grains were carried out with an optical microscope at a magnification of x400, following two random parallel lines for each pollen specimen prepared (Müller 2018), thus a total of 4 lines for each *A. hesperia* specimen. At the end, the whole slide was checked for other pollen morphologies. The percentage of different pollen morphologies was estimated, excluding forms with a frequency below 10% to avoid bias due to possible contamination (Müller & Kuhlmann 2008). Morphological identification of pollen grains was based on online recognition keys and comparison with online image collections, mainly PalDat (Palynological Database; https://www.paldat.org/), DiscoverLife (https://www.discoverlife.org/mp/20q?guide=Pollen) and The Global Pollen Project (https://globalpollenproject.org/). Comparison was also made with the plant species identified in the field near the nests.

Statistical analysis

For microbiota, the correlation between the number of bacterial strains isolated and the sex of the bees was examined by Poisson Generalized Linear Model (GLM).

Pathogen prevalence was computed, and the mean difference in prevalence between newly emerged and free-ranging individuals was examined by Wilcoxon test, as the assumption of normality was not met.

To analyse the number of pathogen copies in the collected specimens, a zero-inflated regression model with a gaussian distribution was used. The log10-transformed pathogen abundance was set as the dependent variable, while sex, pathogen type, and the status of the bee specimens (newly emerged versus free-ranging) were designated as independent additive variables.

Selection of optimal models was guided by the Akaike Information Criterion (AIC). A significance level of 0.05 was applied for hypothesis testing. All data analysis and graphical visualization was performed using R version 4.3.2, along with the ggplot2, glmmTMB and lme4 packages (Wickham 2009; Bates et al. 2015; Brooks et al. 2017).

Results

Taxonomic and molecular identification of *Andrena hesperia*

All the *Andrena* individuals emerging under the cages were identified as belonging to *Andrena hesperia* Smith, 1853. Two collected individuals, belonging to both sexes, are displayed in pictures (Fig. 2).

Concerning molecular analyses, *mt*DNA was successfully amplified and sequenced from the specimens. Identical sequences were obtained by the two methods from *A. hesperia* specimens. BLAST analysis confirmed high similarity to a European *A. hesperia* specimen [Accession number MZ867990 (Query cover: 99%; E-value: 0.0; Percentage of identity: 100%)] sampled in 2019 (Weekers et al. 2022). The sequences were deposited in GenBank (OR912556 and OR912557). A phylogenetic tree was constructed using other *A. hesperia* sequences and *A. fulvago*, the other *Chrysandrena* occurring in Italy (Fig. 3).

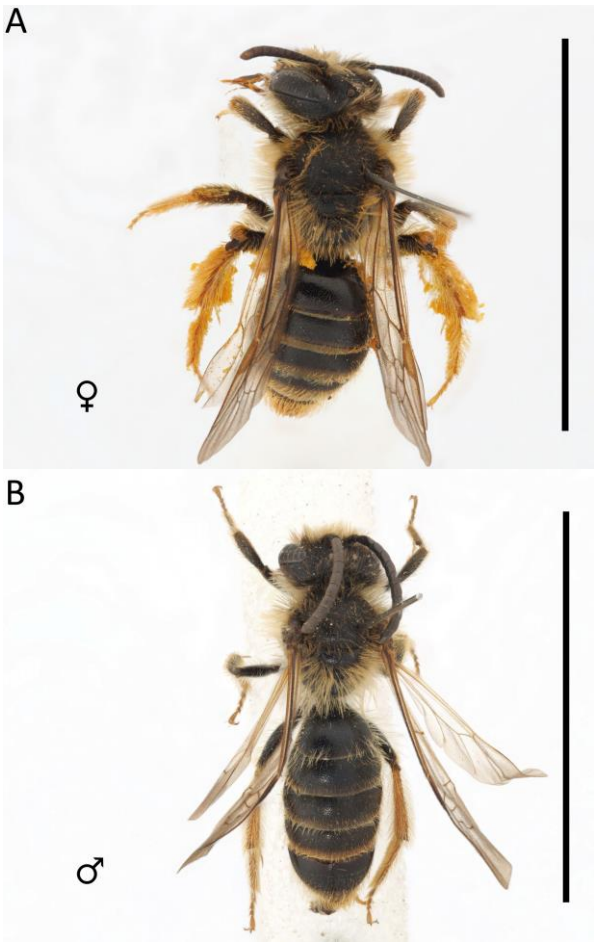

Figure 2. Dorsal view of *Andrena hesperia* female (A) and male (B) collected at the sampling site. Scale bar 10 mm.

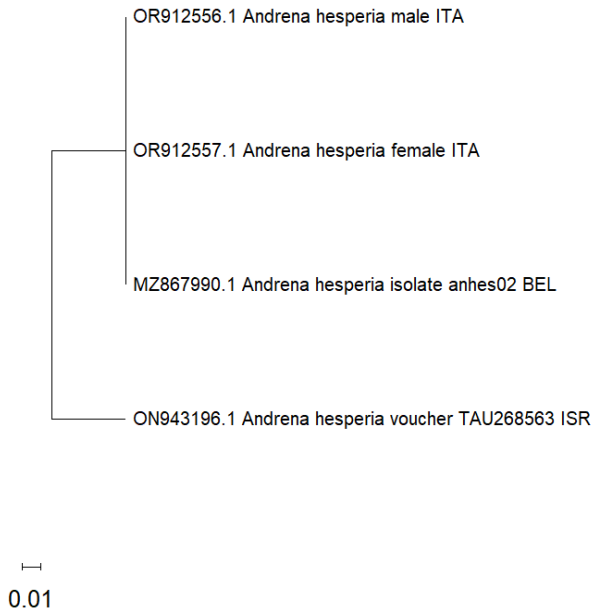

Figure 3. Maximum likelihood nucleotide phylogeny of *Andrena hesperia* and *A. fulvago* sequences deposited in GenBank and BOLD. A sequence of *A. agilissima* was used as outlier. This analysis involved 18 nucleotide sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown above the branches. There was a total of 698 positions in the final dataset. Species and sampling sites are reported for the sequences identified in this study. (FRA: France; FIN: Finland; GBR: Great Britain; ITA: Italy; MOR: Morocco; POR: Portugal; SPA; Spain; TUR: Turkey).

Phenology of Andrena hesperia

A total of 100 males and 48 females of *A. hesperia* were recorded in the emergence cages in the monitoring period. The resulting sex ratio was 0.68 (95% C.I. = 0.61-0.76). The emergence period lasted from 31 March to 18 April 2023 (Fig. 4). Males emerged from nests throughout the entire period, with a daily average of 3.2 ± 5.0 s.d. and a maximum of 19 males per day. The number of males appearing in the cages showed two peaks separated by a pause, the first peak around 4 April and the second around 12 April. Females first appeared three days after males, on 3 April, continuing until 18 April, with a daily average of 3.0 ± 6.0 over the period. They peaked in abundance on 11 April with 23 emerging females. The flight period of males lasted for about ten days after emergence, whereas the activity of females around the nests continued for about a month, ceasing completely by the second half of May, after which *A. hesperia* was not observed for the rest of the year.

Heat stress resistance

The survival time of specimens held at a constant temperature of 40°C averaged 42.53 minutes (decimal format) with a standard deviation of 13.77 (Fig. 5).

Brood parasites and parasitoids

During the monitoring period, one parasitic bee and seven flies were found in the cages. The only brood parasite bee associated with *A. hesperia* nests we found, was a single specimen of *Nomada facilis* Schwarz, 1967. BLAST analysis confirmed its

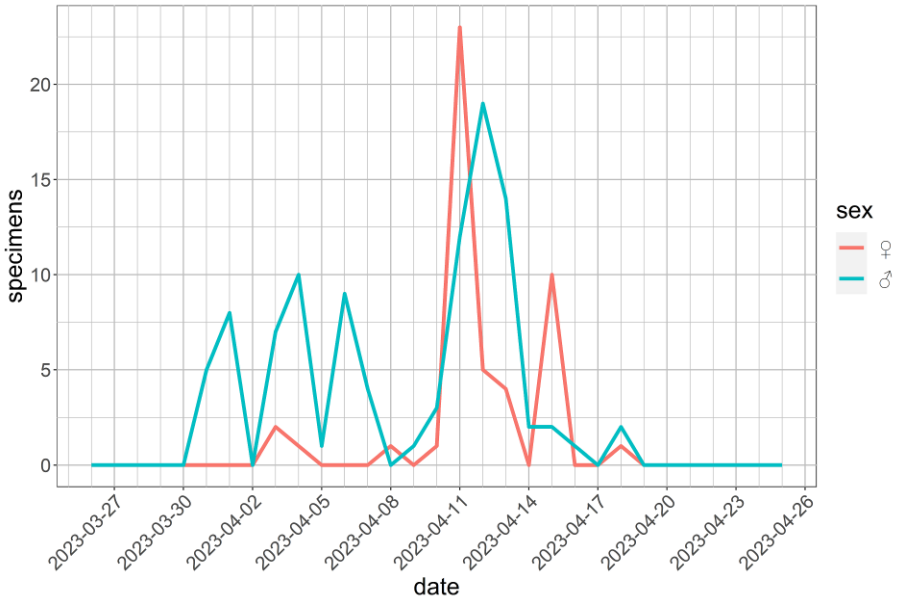

Figure 4. Number of male (blue line) and female (orange line) *A. hesperia* individuals captured in emergence cages during the monitoring period (31 March – 18 April 2023)

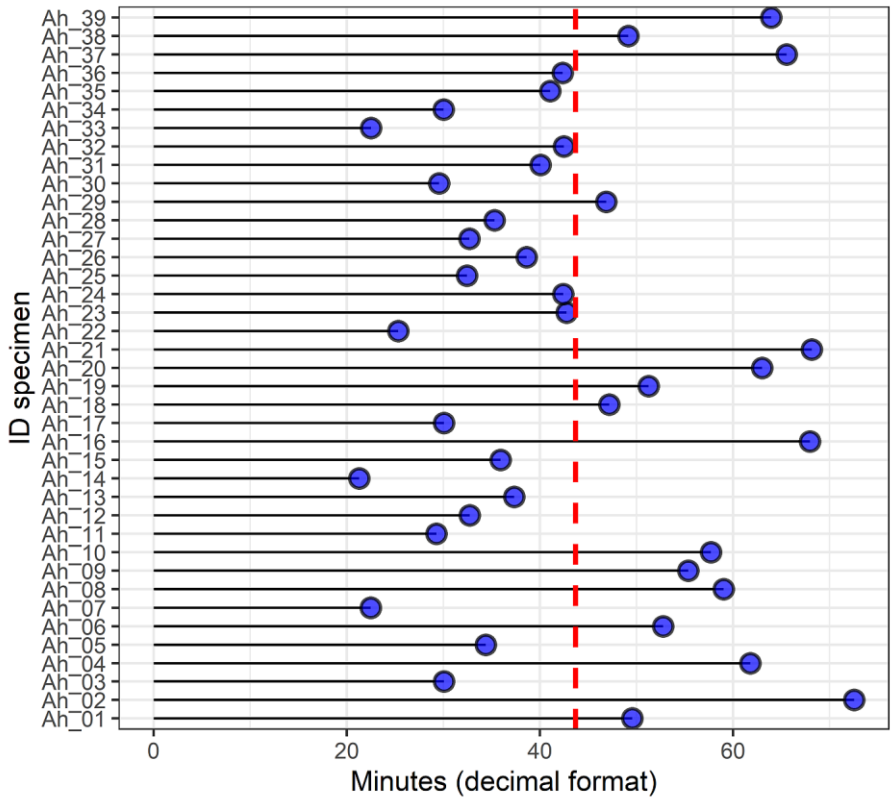

Figure 5. Plot showing the moment when heat stupor was recorded for each bee. The dashed red line represents the average resistance time.

similarity (99.85%) to Italian (sampled in the Aosta Valley region) and German (sampled in Baden-Wuerttemberg) specimens of *N. facilis* (Accession numbers, HQ948027, KJ837786, HM401069, HM401068) (Schmidt et al. 2015). The sequence was deposited in GenBank (PP198887).

Three flies were identified as *Bombylius canescens* Mikan, 1796 (Diptera: Bombyliidae), two as *Pegomya* Robineau-Desvoidy, 1830 (Diptera: Anthomyidae), related to the *P. terminalis* (Rondani, 1866) species group, and one as *Delia platura* (Meigen, 1826) (Diptera: Anthomyidae). The seventh was a fly of the genus *Bellardia* Robineau-Desvoidy, 1863 (Diptera: Calliphoridae).

Pathogen characterization of *A. hesperia*

The pathogens detected were DWV, CBPV, ABPV, KBV, *AmFV, N. ceranae* and *C. bombi.* The highest prevalence was recorded for DWV (76.27%), followed by *N. ceranae* (49.15%), CBPV (30.51%) and *C. bombi* (10.17%). The Wilcoxon test showed a significant difference in pathogen prevalence, free-ranging specimens showing a higher prevalence of these pathogens than newly emerged *A. hesperia* (W = 64, *p value* = 0.038). Interestingly, the other five viruses (ABPV, KBV, SBV, BQCV and *Am*FV*)* were only detected in free-ranging adults (Fig. 6).

The Zero inflated GLM employed to model the data and estimate pathogen abundance in samples of different sex and bee status (Fig. 7) showed a strong negative correlation between pathogen abundance and newly emerged individuals. *Nosema ceranae* is the only pathogen that shows a significant effect on the dependent variable. No significant effects were found for the sex variable (Table S1). Overall, 58% of the model variance of the dependent variable was explained by independent variables.

Gut microbiota composition

The bacterial community of newly emerged *A. hesperia* was dominated by Bacillota (*Lactobacillus* and *Fructobacillus*) and Pseudomonadota (*Snodgrassella alvei* and *Gilliamella*). More specifically, gut microbiota included *Bacillus licheniformis, B. thuringiensis, Enteococcus durans, Enterobacter aerogenes, Enterococcus faecalis, Fructobacillus fructosus, Gillamella apicola, G. bombi, G. intestine, G. mensalis, Lactobacillus acetotolerans, L. Firm-4, L. Firm-5, L. fructivorans, L. kunkeii, L. lindneri, Pseudomonas aeruginosa, Snodgrassella alvei,*

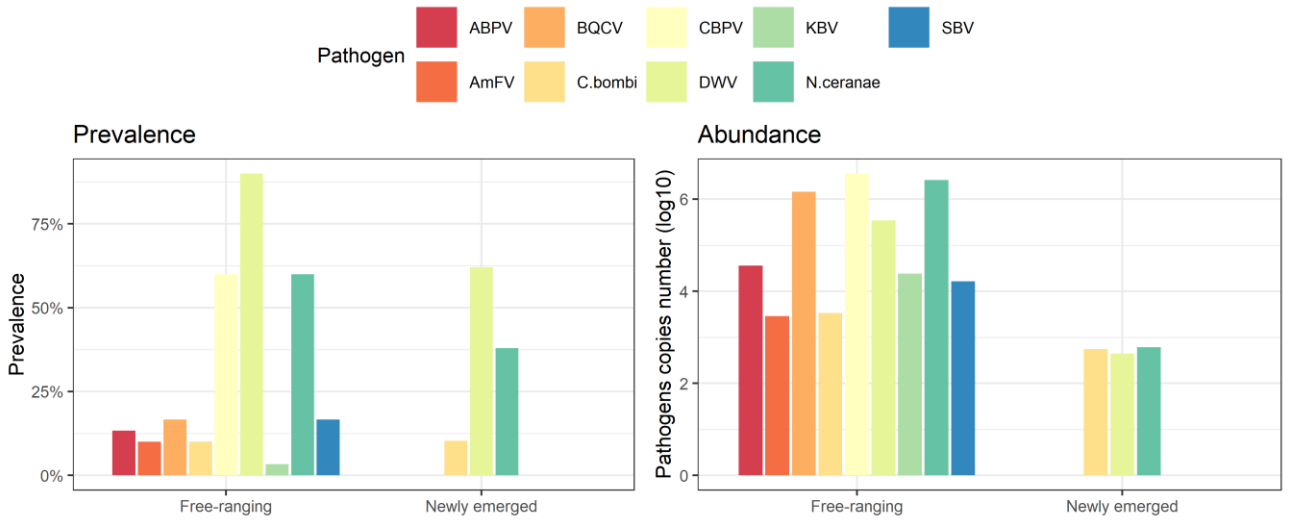

Figure 6. Prevalence and abundance of pathogens detected in *A. hesperia*

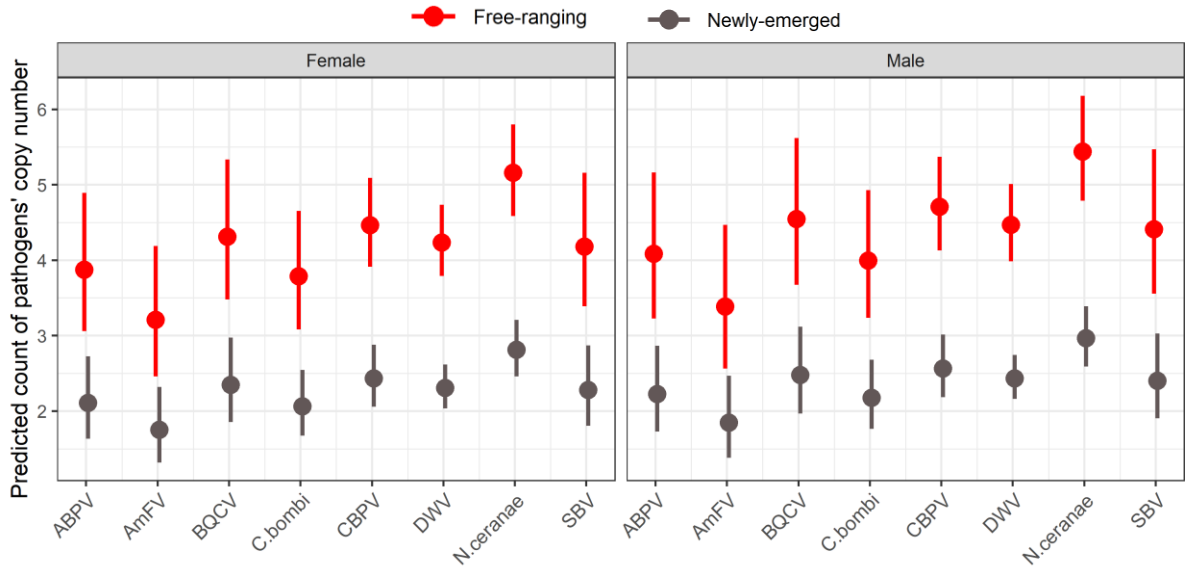

Figure 7. Predicted values for pathogen abundance in *A. hesperia* individuals. Although no differences were observed between males and females, pathogen abundance was significantly higher in free-ranging individuals. *N. ceranae* proved to be the most prevalent pathogen.

Streptococcus epidermidis, S. saprophyticus and *S. thermophilus* (Fig. 8).

Poisson GLM with log link function employed to model the data and estimate the number of microbiota strains (Fig. 9) showed a significant number of *Lactobacillus Firm-4* (GLM estimate = 1.25, se = 0.57, P = 0.03), *Firm-5* (GLM estimate = 1.18, se = 0.57, P = 0.04) and *Snodgrassella alvi* (GLM estimate = 1.25, se = 0.57, P = 0.02) in the samples, unrelated to the variable sex (Table S2). Overall, 63% of model variance was explained by independent variables.

PALYNOLOGICAL CHARACTERIZATION

Pollen grain count was higher in *A. hesperia* captured during nesting at the study site than in individuals from previous years in the CREA-AA collection. Considering the sum of the two lines analysed for each slide and the average of the two slides for each sample, we obtained a total average

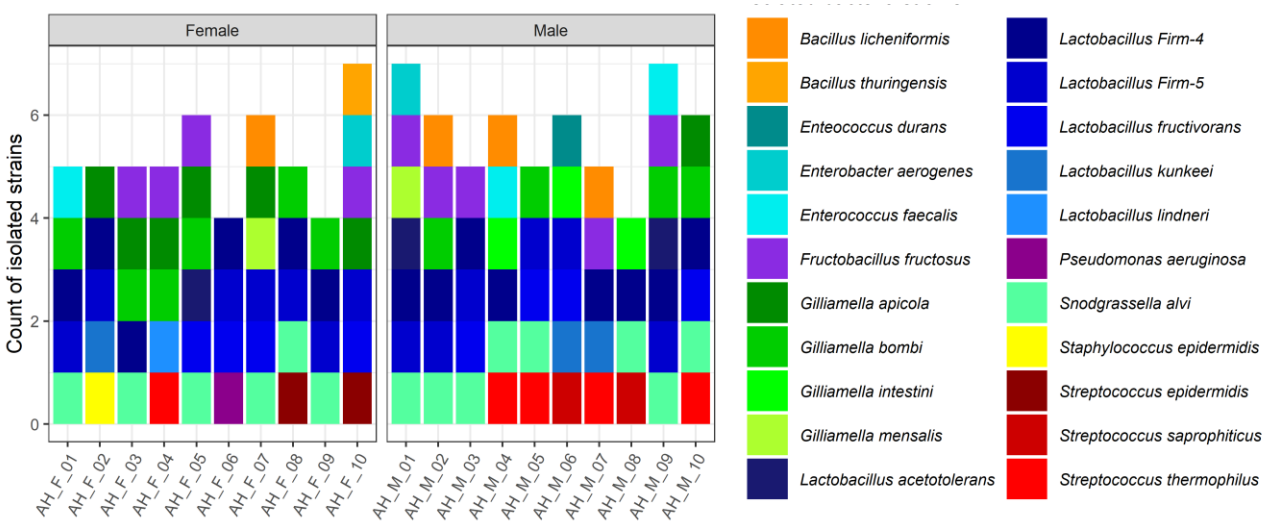

Figure 8. Microbiota strains isolated from the gut of male (M) and female (F) specimens of *A. hesperia* (AH).

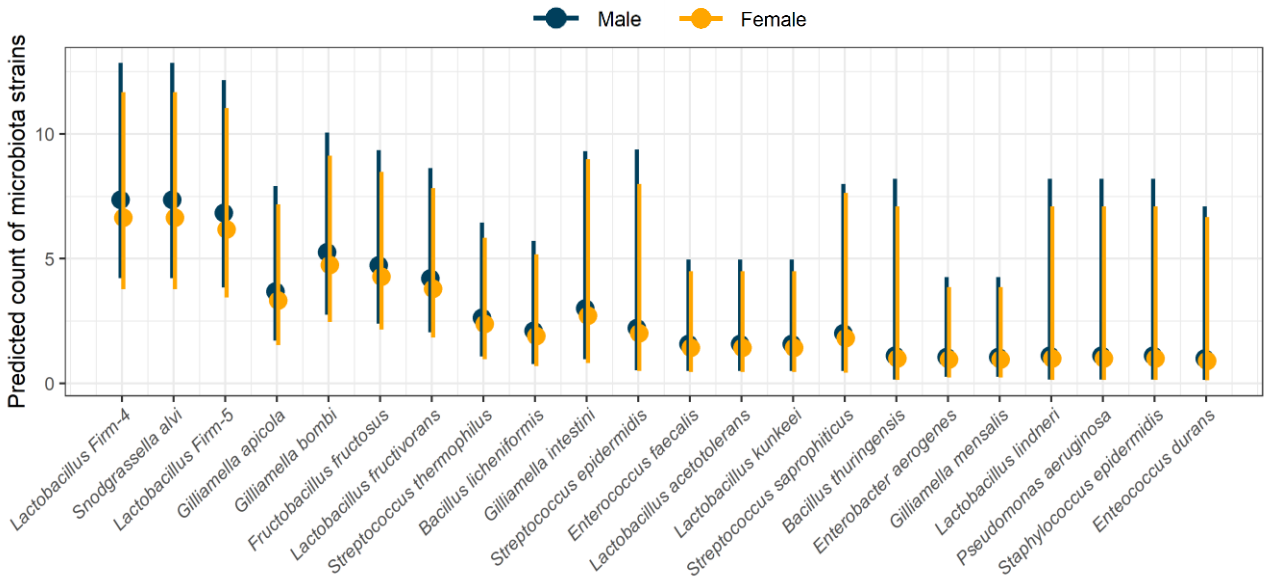

Figure 9. Predicted values of microbiota strains in male and female specimens of *A. hesperia* reveal a preponderance of *Lactobacillus Firm-4*, *Firm-5* and *Snodgrassella alvi*. These particular strains showed a significant prevalence in male individuals.

of 470 pollen grains in fresh samples, compared to 92 pollen grains from collection specimens. The significant difference was due to the pollen extraction method, which showed individual washing to be more effective than manual detachment from bee brushes. However, although washing bees is easier and more effective, it overestimates the quality and quantity of pollen collected to feed bee larvae. Indeed, by washing the entire individual, pollen that the insect gathered accidentally while feeding on nectar may be included. The percentage of this incidental pollen should be less than that collected from the scopae and therefore should not influence the results. To limit this contamination, specimens of *A. hesperia* were captured immediately after they were observed to collect pollen from flowers.

The method of sample collection and conservation also influenced the results: specimens taken from the nesting site were

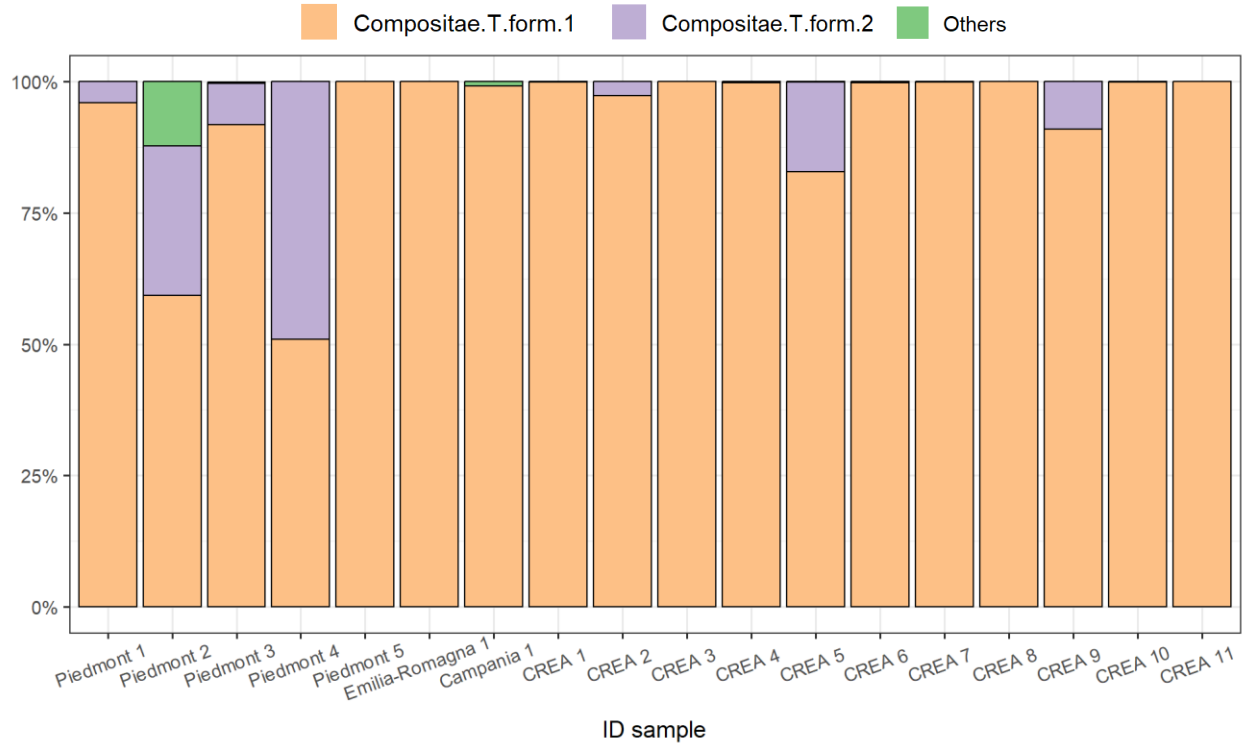

Figure 10. Pollen grain composition by specimen. ID CREA indicates bees from the Bologna population; the others are the seven specimens from the CREA-AA entomological collection.

Figure 11. *Andrena hesperia* female collecting pollen on *Crepis* sp.(A), searching for the nest (B), and entering the nest with her pollen load (C).

captured and preserved individually in small sterile tubes, whereas samples from our collection were captured and placed in larger vials containing cork shavings. This second method presumably caused dispersal of pollen.

Morphological identification found a prevalence of pollen from the Asteraceae family in all samples. Two forms were identified: T1 attributed to the genera *Crepis* and *Hieracium* (Fig. 11) and T2 to the genus *Taraxacum*. Both pollen types belong to the subfamily Cichorioideae. Association with plant genus was confirmed by direct field observations and identification of plant species near *A. hesperia* nests. We also found a small percentage of pollen grains from the Fabaceae, Poaceae and Rosaceae families. Pollen grain percentages below 10% were disregarded as negligible (Fig. 10 and Table S3).

DISCUSSION

A nesting aggregation of *Andrena hesperia* in the courtyard of CREA Research Centre offered the opportunity to study the basic traits of the species and its relationships with other organisms. *Andrena hesperia* cannot be considered a rare species and is classified *Least Concern* by the

European Red List of Bees (Nieto et al. 2014). It has a wide distribution, ranging from Central Asia to southern Europe and the Mediterranean (Amiet et al. 2010; Comba 2019). Italian reports of the species are rare and come from only nine of the 20 Italian regions, despite the fact that the species was originally described from Italy (Smith 1853). Although the species was already known from the neighbouring regions of Lombardy, Liguria and Tuscany (Quaranta et al. 2004; Comba 2019), this is the first report of *A. hesperia* from Emilia-Romagna. This situation and the fact that most of Italy is potentially suitable and in the centre of the geographic range of *A. hesperia* (Smith 1853; Gusenleitner & Schwarz 2002), suggests that more information is needed even for this relatively common species.

The nest aggregation does not appear to be hosting a proportionate number of parasitic insects. The only brood parasite bee collected was a single specimen of *Nomada facilis*. Although little information is available about its hosts, this species has been associated with *Andrena fulvago*, another species of the subgenus *Chrysandrena* that is also specialised in collecting pollen from Asteraceae subfamily Cichorioideae (Notton & Norman 2017).

Of the four species of flies that emerged in the cages, only *Bombylius canescens* can be associated with *A. hesperia*. *B. canescens* is known to parasitize a wide spectrum of hosts, including bees of the genera *Halictus*, *Lasioglossum* (Halictidae) and *Andrena* (Andrenidae) and wasps of the genus *Odynerus* (Vespidae) (Boesi et al. 2009). It is therefore extremely likely that the *Bombylius* collected came from *A. hesperia* nests, unlike what can be assumed for the other species of fly. The two Anthomyiidae, *Pegomya* sp. and *Delia platura* develop from larvae with a phytophagous diet on Equisetaceae and Fabaceae respectively (Tai & Hyung 1989; Michelsen 2008). On the other hand, the genus *Bellardia* contains flies that are parasites of earthworms (Rognes 1991). In neither case can any link with *A. hesperia* yet be hypothesized.

The emergence period of the nesting population of *A. hesperia* at CREA spanned 19 days from the end of March to mid-April. The flight period continued for a month and a half until mid-May, suggesting typical univoltine phenology. Across its entire geographic range, the flight season of the species is longer, from the beginning of March to the end of July (Amiet et al. 2010). Note that the number of bees emerging varied greatly from day to day, possibly due to local weather conditions, though a definite explanation has yet to be found. The population proved to be protandrous, with many male bees appearing throughout the whole period, while female bees only became abundant in the second half of the period. Protandry is a common feature among solitary bees with monogamous females that tend to mate as soon as they emerge (Danforth et al. 2019). The sex ratio of emerging bees was remarkably male-biased, in contrast to information about other *Andrena* species that show a more female-biased sex ratio (Paxton & Tengö 1996; Paxton et al. 1999; Lund Norbakk 2017). Unfortunately, the limitations of the study do not allow a satisfactory explanation of this phenomenon. Nevertheless, first-hand data on bee phenology and life cycle will be fundamental in the foreseeable future. Such data will be useful for tracking trends related to climate change, which is already inducing a shift in wild bee phenology (Dorian et al. 2023).

Due to the increase in heatwaves associated with climate change, there has been recent scientific interest in protecting pollinators (Biella et al. 2022; Herrera et al. 2023). Wild bees seem to be highly susceptible to these changes, also in relation to altered flower phenology (Rafferty 2017; Schenk et al. 2018).

It has been observed that species with a limited distribution appear to be more sensitive to thermal stress, even though species not accustomed to high altitudes are more resistant (Martinet et al. 2015, 2018; Oyen et al. 2016). In the case of male bumble bees, the time before heat stupor ranges from 27 to 474 minutes along a latitudinal and elevation gradient. Bumble bee specie whose distribution is centred on the Mediterranean displayed the longest THS, and alpine and arctic species the shortest (Martinet et al. 2021). *Andrena hesperia* wide distribution range covers the entire Mediterranean, from the Middle East to European and North African coasts (Bossert et al. 2022). Nevertheless, the time before heat stupor of *A. hesperia* females (42.53 ± 13.77 minutes) is lower than the ones detected for female bees (Apidae and Megachilidae) of the Lebanese fauna (Boustani et al. 2024). This could suggest that *A. hesperia* is less

adapted to a hot Mediterranean climate. The modest tolerance to high temperatures may be explained by *A. hesperia* being one of the first species to emerge post-winter in Italy, with mild temperatures still present.

Interspecies transmission of pathogens in bees is well documented (Dalmon et al. 2021; Pritchard et al. 2021; Nanetti et al. 2021a). The main routes are via flowers, infection occurring during foraging or by direct contact with infected individuals (Yañez et al. 2020; Burnham et al. 2021; Fearon & Tibbetts 2021; Cohen et al. 2021). As for other *Andrena* species, DWV was the most prevalent and abundant pathogen and ABPV, BQCV, SBV and *N. ceranae* were also detected. *Am*FV, CBPV, KBV and *C. bombi* were detected for the first time in *Andrena* species. In fact, DWV, ABPV, SBV, BQCV, CBPV and KBV are widespread in wild and honey bees and other hymenopterans, showing greater abundance and higher prevalence (Porrini et al. 2016; Forzan et al. 2017; Mazzei et al. 2018; Paxton et al. 2022; Bordin et al. 2022; Cilia et al. 2022b; Maggio et al. 2024). In the same sampling site, *Am*FV was detected in hymenopterans nesting in a bee hotel (Cilia et al. 2023a) and SBV, CBPV and KBV were found in hymenopteran samples from Emilia-Romagna and surrounding regions (Mazzei et al. 2019; Cilia et al. 2022b, 2022c; Zucca et al. 2023). The detection of DWV, *C. bombi* and *N. ceranae* in newly emerged individuals suggests that infection may have occurred through contaminated pollen fed to the larvae (Chen et al. 2006; Mockel et al. 2011; Mazzei et al. 2014; Rothman et al. 2019). As for most wild bee species (Mazzei et al. 2014; Dolezal et al. 2016; Tehel et al. 2020; Gómez-Moracho et al. 2021; Zhang et al. 2021; Tiritelli et al. 2024), the effects of pathogens on *A. hesperia* are unknown and there is insufficient data to define the real impact of pathogens on bee communities.

The microbiota of newly emerged females and males of *A. hesperia* did not show significant differences, being mainly composed of lactic acid bacteria and Enterobacteriacea. These main bacteria strains were also detected previously in *A. vaga* (Hettiarachchi et al. 2023). Some Bacillota (*L. acetotolerans, L. Firm-4, L. Firm-5, L. fructivorans, L. kunkeii, L. lindneri* and *F. fructosus*) and Pseudomonadota (*G. apicola, G. bombi, G. intestine, G. mensalis* and *S. alvei*) belong to the strains present in the gut of honey bees, bumblebees and other bee species, suggesting that these bacteria circulate in the environment during foraging (Engel et al. 2012; Keller et al. 2013; McFrederick et al. 2017; Kwong et al. 2017; Voulgari-Kokota et al. 2019a; Nguyen & Rehan 2023). Other bacteria detected (*Bacillus, Enterococcus, Enterobacter, Streptococcus* and *Pseudomonas*) may be environmental contaminants (Cilia et al. 2023b) or could favour larval growth and enzymes for digesting pollen (Dharampal et al. 2019, 2020; Steffan et al. 2019; Voulgari-Kokota et al. 2019b; Keller et al. 2021). Microbes are acquired by solitary bees primarily through foraging preferences and nesting behaviour (McFrederick et al. 2017; McFrederick & Rehan 2019; Rothman et al. 2019; Nguyen & Rehan 2023). Pollen provisions, regulated by the plants foraged and the surrounding environment, and influenced by floral resources, are important (McFrederick et al. 2017; Voulgari-Kokota et al. 2019b; Dew et al. 2020; Chui et al. 2022; Nguyen & Rehan 2023). For ground-nesting bees, some bacterial strains are useful for the metabolic conversion and preservation of larval provisions or provide enzymes involved in protein, lipid and carbohydrate catabolism (Gilliam et al. 1984, 1990; Keller et al. 2013; Voulgari-Kokota et al. 2019a; Voulgari-Kokota et al. 2019c; Nguyen & Rehan 2023).

Considering our pollen analysis of *A. hesperia* samples, the univoltine life cycle confirmed by our findings, allowing for limited flight time and consequent high competition for resources (Wood & Roberts 2018), it can be concluded that *A. hesperia* is oligolectic for Asteraceae (Cichorioideae), in line with current knowledge for all members of the subgenus *Chrysandrena* (Amiet et al. 2010; Wood 2023). Although there were many flowering individuals in the sampling area in spring, including species of the genera *Veronica*, *Ranunculus* and *Bellis*, the bees showed an almost total preference for the genera *Crepis*, *Hieracium* and *Taraxacum* of the Cichorioideae subfamily. This aligns with the findings of Dermane and colleagues (2021), whose results indicate an absolute preference for species of the Asteraceae family. Plants have evolved adaptations that set a balance between pollinator attraction and pollen loss (Westerkamp 1997; Westerkamp & Claßen-Bockhoff 2007). These adaptations include both

morphological and chemical traits. Asteraceae pollen walls feature many thick layers and have good mechanical resistance (Meier-Melikyan et al. 2003). The pollen also has chemical properties that interfere with pollen digestion and proper development of larval stages, a phenomenon exclusive to generalist bees (Praz et al. 2008). Oligolectic bees, in turn, have evolved physiological adaptations to counteract plant defence mechanisms that limit pollen availability (Singer 2008; Sedivy et al. 2011; Wood & Roberts 2018). It therefore seems likely that *A. hesperia* has evolved physiological adaptations that allowed it to develop a preference for pollen that is typically difficult to digest, thus favouring specialization for Asteraceae. Further studies on the physiology and trophic preferences of the species are needed to investigate these hypotheses.

Conclusion

The present study will hopefully set the basis for better knowledge of the bionomics of *Andrena hesperia* and shed light on its relationships with other organisms. First-hand phenology data is needed to evaluate the effects of climate change on the seasonality of the species. To better understand the effect of climate change, it will be necessary to repeat the investigation on the phenology of *A. hesperia* in future years and in other parts of its geographic range. The effects of pathogens on *A. hesperia* and their impact on bee communities remain to be discovered. Further studies on fitness, behaviour and development are needed to understand epidemiological features. Many bacterial strains are shared with other wild bees, bumblebees and honeybees, suggesting that they are exchanged in the environment during foraging. In general, knowledge of *Andrena* microbiota needs to accrue in order to understand the relationship of the species with the environment and the effect of fitness. Unfortunately, little has been published on this for other species of wild bees. Basic research, focused on the bionomics of single species, is essential for investigating the ecology of wild bee communities faced with anthropogenic factors like climate change, habitat loss and pesticide use.

Acknowledgements

We thank dipterists Daniele Birtele (Bombyliidae), Verner Michelsen (Anthomyiidae), Daniele Avesani and Giuseppe Lo Giudice (Calliphoridae) for helping us with the identification of flies.

Author contribution

Concept and design GC, MdA & SF; data collection GC, ELZ, MdA, LZ, RR & SF; formal analysis GC & RR; data analysis ELZ, MdA & LZ; species identification SF; writing GC, ELZ, MdA, LZ, RR & SF, edits and approval for publication GC, ELZ, MdA, LZ, RR, LB & SF. Funds acquisition LB.

Disclosure statement

No potential conflict of interest was reported by the authors.

Data availability statement

The COI sequences are available in GenBank (Accession Number OR912556, OR912557 and PP198887).

Appendices

Additional supporting information may be found in the online version of this article:

Table S1. Model estimate, standard error (SE), and P value of Zero-Inflated GLM.

Table S2. Model estimate, standard error (SE), and P value of Poisson GLM.

Table S3. Percentage of pollen grains composition for each analysed specimen.

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 ISSN 1920-7603