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First records of *Anagraphis ochracea* (Araneae: Gnaphosidae) for continental Italy and Sicily with new observations on its myrmecophilous lifestyle

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Abstract. In the present study we describe and discuss for the first time the peculiar myrmecophilous habits of *Anagraphis ochracea* (L. Koch, 1867) and its strong association with the ant species *Messor ibericus* Santschi, 1931. The study is based on behavioural observations carried out both in the field and in captivity, and sheds light on the lifestyle of this poorly studied and rarely observed species. We also recorded the presence of *A. ochracea* on continental Italy and Sicily for the first time; provide a brief overview of its taxonomical history and present photographs of adult and juvenile specimens, the egg sac and the copulatory organs of both sexes. Finally, we provide a DNA-barcode (COI) for *A. ochracea,* which is the first for the genus *Anagraphis* as well.

Keywords: ant, ant association, Arachnida, *Messor ibericus*, myrmecophily, spider, symbiosis

Zusammenfassung. Erster Nachweis von *Anagraphis ochracea* (Araneae: Gnaphosidae) für das italienische Festland und Sizilien, mit neuen Beobachtungen zum myrmekophilen Lebensstil der Art. In der vorliegenden Studie wird zum ersten Mal das besondere myrmekophile Verhalten von *Anagraphis ochracea* (L. Koch, 1867) und ihre enge Bindung an die Ameisenart *Messor ibericus* Santschi, 1931 beschrieben und diskutiert. Die Studie basiert auf Beobachtungen zum Verhalten im natürlichen Lebensraum wie auch im Labor und gibt Aufschluss über die Lebensweise dieser wenig erforschten und selten gefundenen Art. Ebenso wird der Erstnachweis von *A. ochracea* für das italienische Festland und Sizilien erbracht, sowie eine Zusammenfassung der taxonomischen Historie, Bilder adulter wie auch juveniler Tiere, der Kopulationsorgane beider Geschlechter und des Eikokons präsentiert. Zusätzlich wird erstmals der genetische Barcode (COI) der Art, und auch der Gattung *Anagraphis*, beschrieben.

The Italian spider fauna is amongst the most diverse in all of Europe (Nentwig et al. 2022). However, the description of multiple new taxa and the recurring identification of previously unrecorded species keep enriching knowledge of the known biodiversity. Currently, 53 spider families are represented in Italy, for a total of 440 genera and 1700 species (Pantini & Isaia 2019). Among these, Gnaphosidae is the second most diverse family in the country, with a total of 169 recorded species and 31 genera.

Anagraphis Simon, 1893 is a small genus represented by only seven known species distributed in the Palaearctic and Afrotropical regions of the world (World Spider Catalog 2022). Several of these taxa are quite obscure, having been superficially described in the first half of the 20th century and largely ignored ever since. *Anagraphis ochracea* (L. Koch, 1867) is a poorly studied and rarely observed species, originally described as *Liocranum ochraceum* from Corfu, Greece, and currently recorded also for Albania, Northern Macedonia, Turkey, the Russian Caucasus and Sardinia (World Spider Catalog 2022).

Until now, studies on the genus *Anagraphis* and, more specifically, on *A. ochracea*, have focused exclusively on taxonomy and distribution. Most of the more recent contributions have concentrated only on Aegean species (Chatzaki et al. 2002a, 2002b), and have centered their work on preserved museum material and dead specimens collected in the field. No author has ever provided behavioural or ecological notes for *A. ochracea* or any similar, congeneric species.

Myrmecophily, or the tendency of certain species to live within or near ant nests in strong symbiotic association with ants (Donisthorpe 1927), has been observed in only 13 of the 132 known spider families, for a total of 41 different species (Cushing 1997, 2012). Fourteen of these are currently recorded for Italy (Pantini & Isaia 2019). Although several authors have noted and studied this peculiar behaviour within the Araneae (Hölldobler & Wilson 1990, Cushing 1997, 2012, Witte et al. 2008, Nelson & Jackson 2009), most have primarily focused on myrmecomorphy (mimcry of ants) and myrmecophagy (the consumption of ants). As of today, very few contributions have discussed myrmecophily and the social integration of spiders in ant colonies (Mendonça et al. 2019). Several myrmecophile spider species not only resort to active, evasive manoeuvres to avoid direct interaction with the ant, but have also developed the ability to chemically hide. Some absorb, biosynthesise or imitate the cuticular hydrocarbon profile of the hosts in order to survive unscathed within the ant colony (Cushing 2012). Others reduce the emission of cuticular hydrocarbons in order to remain unnoticed (Parmentier et al. 2017). Given the very limited number of contributions focusing on the subject (e.g., Castellucci et al. 2022), the study of myrmecophile spiders in Italy is a largely unexplored field.

Here, we record and report the presence of three distinct populations of *A. ochracea* in south-central Italy. Field sampling and extensive observations were carried out, both in the

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field and in the lab, to thoroughly investigate its behaviour. Significant and previously unrecorded behavioural information was obtained. We report for the first time that *A. ochracea* is strongly associated with the ant species *Messor ibericus* Santschi, 1931, and lives unscathed and unnoticed within their colonies.

The focus of this work is thus to present a series of observations that describe *Anagraphis*'s unreported myrmecophilous lifestyle and the presence of a new species in continental Italy and Sicily. Furthermore, we provide high quality photos of the habitus and genitalia to further facilitate its identification, publishing the very first contribution towards the genetic characterization of both the target species and the genus *Anagraphis* as a whole.

Materials and methods

During the course of this study, more than 40 different live specimens were observed and analysed, both in the wild and in the lab. The very first hint of the strong myrmecophilous habit of *A. ochracea* was observed and reported by Luigi Lenzini in populations from the Lazio region.

Subsequently, similar observations were carried out by Mattia Poso in southern Italy, specifically in the area around Lecce. All specimens were preserved in either 75% or 96% ethanol. Over the years, several specimens encountered in the Caffarella Valley were collected from the field and held in captivity by L. Lenzini for additional behavioural observations. The spiders were raised or kept individually in 5cm wide, squared glass enclosures covered by an opaque liq. The setup was simple and comprised a layer of mixed terrain intended to recreate the natural substrate found in the field near ant colonies. Enclosures were kept in a dimly lit environment while water was sporadically sprinkled to simulate a rainy day and to provide a degree of humidity to the substrate.

Specimen identification and morphological analysis was carried out utilizing both a Zeiss Stereomicroscope II and a Leica MZ16. The photos of preserved specimens were obtained by connecting an Olympus E-M1 or an Olympus E-M5mkII to the microscopes. Photos of the habitus and of live specimens in the field were obtained using the following cameras: Panasonic Lumix FZ28, FZ48, FZ200+ Raynox DCR250, Olympus E-M1 or Olympus E-M5 mkII equipped with a Zuiko 60mm f2.8 + Raynox DCR250. Photos were stacked with Helicon Focus (Version 7.0.2) and processed in Adobe Photoshop CC 2018.

The artificial formicarium used to simulate a natural setting in the lab was purposely constructed in plexiglass by Valerio Dolci. The structure consists of a wide outside arena and a series of interconnected and sub horizontal, underlying chambers. After having observed and confirmed a clear degree of mutual tolerance between *A. ochracea* and *M. ibericus* under lab conditions, several other species of spiders were introduced to test whether the same ant colony would react differently to these spiders. A various number of specimens of *Filistata insidiatrix* (Forsskål, 1775) (Filistatidae), *Lycosoides coarctata* (Dufour, 1831) (Agelenidae), *Steatoda nobilis* (Thorell, 1875) (Theriidiidae), and *Scotophaeus blackwalli* (Thorell, 1871) (Gnaphosidae) were utilized. These were selected among non-myrmecophilic species that are commonly found in the same area as *A. ochracea* in the Caffarella Park and being of comparable dimensions to *A. ochracea*.

Genomic DNA was extracted from two legs of each specimen using the NucleoSpin® DNA Insect kit (Macherey-Nagel) and following the manufacturer's instructions. A partial segment of gene loci cytochrome oxidase subunit I (COI) was targeted for PCR amplification using the primer pair LCO1490-HCO2198 (Folmer et al. 1994) as per protocol established by Wheeler et al. (2017). PCR products were utilized for DNA electrophoresis on a 1% agarose gel and purified, prior to sequencing, using the ExoSAP-IT Product Cleanup Reagent (Thermo Fisher Scientific). Sanger sequencing for both forward and reverse reads was performed by Macrogen Europe (Amsterdam, Netherlands). Chromatograms were read using the SeqTrace v.0.9.0 software, while a BLASTn (Zhang & Madden 1997) search was run on the NCBI database to test for possible contamination. The resulting sequence was submitted to GenBank.

All information pertaining to the annual weather and rainfall of the sampled localities was obtained from the WorldClim 2.1 database (https://www.worldclim.org/). Specimens observed during this study are preserved and deposited in the following institutions: MNHT, Museum of Natural History of Trieste, Trieste, Italy; MSNS, Natural History Museum of the Salento, Calimera, Italy; NHMB: Natural History Museum, London, UK; ZMUA, Zoological Museum of the University of Athens, Greece; NHMC, Natural History Museum of Crete, Greece.

Taxonomy and distribution

Anagraphis ochracea **(L. Koch, 1867)** (Figs 1, 3)

For complete taxonomic references, see World Spider Catalog (2022)

Type material. Holotype ♀ of *Liocranum ochraceum* from Corfu (GREECE): NHMB (MB b842); examined. Well preserved and in good condition.

Holotype ♂, paratype ♀ of *Talanites pallidus* from Attica (Attiki), Penteli-Dionysos and Parnis-Phyli (GREECE): Coll. Hadjissarantos (ZMUA). Not examined. The type series could not be found in the ZMUA museum collection or in any other Greek collection (C. Georgiadis, E. Valakos, M. Chatzaki pers. comm.) and is presumed lost.

Examined material. ITALY: Lazio: Rome (RM), Parco Regionale dell'Appia Antica, (41.862681°N, 12.514195°E): 1 ♀, 25. Feb. 2012, L. Lenzini (MNHT); 1 ♂, 14. Jul. 2012, L. Lenzini (MNHT); 1 ♂,1 ♀, 18. Apr. 2018, L. Lenzini (MNHT); 3 ♂♂, 3 ♀♀, 15. May 2018, L. Lenzini (MNHT); 2 ♂♂, 11. Jun. 2018, C. M. Legittimo (MNHT); 1 ♀, 20. Feb. 2019, L. Lenzini (MNHT); 2 ♂♂, Feb. 2019, L. Lenzini (MNHT); 1 ♂, 15. Jun. 2019, L. Lenzini (MNHT). Apulia: Lecce (LE), fraz. Solicara, (40.429889°N, 18.173889°E): 1 ♀ subadult, 16. Aug. 2018, M. Poso (MNHT); 1 ♀, Mar. 2019, M. Poso (MNHT); 1 ♂, 12. Apr. 2019, M. Poso (MNHT); 1 ♂, 19. Apr. 2019, M. Poso (MSNS); Sicily: Buccheri (SR), (37.124667°N, 14.848194°E): 1 ♂, 29. Sep. 2020, G. Romagna (MNHT).

GREECE: Trikala: Antichasia: 1 ♀, May – Jun. 2011, (NHMC FC 11602); Pieria: Litochoro: 1 ♂,19. Jun. – 9. Aug. 2014, (NHMC FC 8798); Magnesia: Alikes: 1 ♂, 23. Apr. – 27. Jun. 2014, (NHMC FC 9276); Portaria to Chania: 1 ♂, 24. Apr. – 28. Jun. 2014, (NHMC FC 9281); Afetes: 2 ♂♂, 1 ♀, 25. Apr. – 29. Jun. 2014, (NHMC FC 9283); Genati: 1 ♀, 25. Apr. – 30. Jun. 2014, (NHMC FC 9284); Samos

Fig. 1: *Anagraphis ochracea*. **a.** habitus of adult female; **b.** habitus of adult male; **c.** preserved female under microscopic light; **d.** closeup of distinctive abundance of long setae

island: Psili Ammos: 1 ♂, 6. Jul. – 30.Jul. 2006, (NHMC FC 9214); Corfu: Corfu: 1 ♀, Josef Erber, Holotype (NHMB BM b842).

Additional material examined

Anagraphis pallens **Simon, 1893**
GREECE: Karpathos, Pigadia-Aperi: 5 ♂♂, 5 ♀♀, 12. May – 23. Aug. 2001 (NHMC FC 1774); Pyles-Volada: 2 ♂♂, 3 ♀♀, 12. May – 23. Aug. 2001 (NHMC FC 1772); Avlona: 1♂, 12. Oct. 2013 (NHMC FC 15973); Achordea: 2 ♂♂, 2 ♀♀, 12. Oct. 2013 (NHMC FC 15974); Rhodes: Afantou-Psinthos: 3 ♂♂, 1 ♀, 14. May – 10. Jul. 2006 (NHMC FC 8461).

Taxonomic history and distribution

In 1867, L. Koch described *Liocranum ochraceum* from an adult female collected on the Greek island of Corfu. In 1940, Hadjissarantos described *Talanites pallides* from two type localities in the Attica region, not far from Athens: Penteli and Fyli. Decades later, Chatzaki et al. (2002b) provided a modern redescription of *T. pallides*, transfered the species to the genus *Anagraphis* and added the Greek isle of Antikythera as a new sampled locality. Lastly, Bosmans (2014) identified *Liocranum ochraceum* as a senior synonym of *Anagraphis pallida* and proposed their synonymy.

The authors of the present work could not locate the type series of *A. pallida*. No specimen was found in the ZMUA collection, and the series is presumed lost. As such, it is not possible to either confirm or refute the validity of the synonymy proposed by Bosmans (2014). However, the holotype of *A. ochracea* and the description provided by Chatzaki et al. (2002b) for *Talanites pallides* do indeed share multiple striking similarities. Bosmans (2014) also proposed the synonymy of *Anagraphis ochraceum* with *Macedoniella karamani* Drensky, 1935, a species originally described from Northern Macedonia. Additional, more recent records of *A. ochraceum* have been made in Turkey (Demircan & Topçu 2015) and Sardinia (Caria et al. 2021). A single female record from the Russian Caucasus (Ponomarev & Shmatko 2020) needs further confirmation (Nentwig et al. 2022).

During this study, three distinct Italian populations of *Anagraphis ochracea* were observed and recorded. One, the first, inside the metropolitan area of the capital city of Rome, Lazio; one in the northeastern area surrounding Lecce, Apulia; one near Buccheri (SR) on the island of Sicily (Fig. 2). Numerous specimens were collected and identified, but more than 40 different individuals were observed and photographed in the field (Tab. 1).

Anagraphis ochracea is a small to medium sized spider distinguished by its homogeneous cream-coloured appearance

Tab. 1: A detailed list of the total number of specimens observed in the wild from 2009 to 2022, with the associated ant species. Abbreviations: LL, L. Lenzini; CML, C. M. Legittimo; GM, G. Mascia; MP, M. Poso; GR, G. Romagna.

N°	Locality	Date	Obs.	Ant species	N°	Locality	Date	Obs.	Ant species
1	Roma, Caffarella	20. Jan. 2009	LL	---	27	Roma, Caffarella	08. Nov. 2020	LL	*Messor ibericus*
2	Roma, Caffarella	29. Nov. 2009	LL	*Messor ibericus*	28	Roma, Caffarella	21. Dec. 2020	LL	*Messor ibericus*
3	Roma, Caffarella	25. Feb. 2012	LL	*Messor ibericus*	29	Roma, Caffarella	14. Jan. 2021	LL	*Messor ibericus*
4	Roma, Caffarella	29. Mar. 2012	LL	*Messor ibericus*	30	Roma, Caffarella	25. Apr. 2021	LL	*Messor ibericus*
5	Roma, Caffarella	03. May 2012	LL	*Messor ibericus*	31	Roma, Caffarella	19. May 2021	LL	*Messor wasmanni*
6	Roma, Caffarella	14. Jun. 2012	LL	*Messor ibericus*	32	Roma, Caffarella	04. Oct. 2021	LL	*Messor ibericus*
7	Roma, Caffarella	29. Nov. 2012	LL	*Messor ibericus*	33	Roma, Caffarella	05. Oct. 2021	LL	*Messor ibericus*
8	Roma, OstiaAntica	23. Dec. 2012	LL	*Messor ibericus*	34	Roma, Malagrotta	18. Oct. 2020	LL	*Messor ibericus*
9	Roma, Caffarella	18. Mar. 2014	LL	*Messor ibericus*	35	Roma, Tor Marancia	31. Oct. 2020	LL	*Messor ibericus*
10	Fiumicino, Torrinpietra	28. Oct. 2017	LL	*Messor ibericus*	36	Roma, Caffarella	17. Mar. 2022	LL	*Messor ibericus*
11	Roma, Caffarella	12. Nov. 2017	LL	*Messor ibericus*	37	Roma, Caffarella	05. Apr. 2022	LL	---
12	Roma, Caffarella	19. Nov. 2017	LL	*Messor ibericus*	38	Roma, Caffarella	08. Apr. 2022	LL	*Messor ibericus*
13	Roma, Caffarella	16. Mar. 2018	LL	*Messor wasmanni*	39	Tivoli, Monte Catillo	30. Apr. 2022	LL	*Messor ibericus*
14	Roma, Caffarella	17. Mar. 2018	LL	*Messor ibericus*	40	Lecce, Solicara	Jun. 2018	GM	---
15	Roma, Caffarella	18. Apr. 2018	LL	*Messor ibericus*	41	Lecce, Solicara	24. Jun. 2018	MP	*Messor ibericus*
16	Roma, Caffarella	15. May 2018	LL	*Messor ibericus*	42	Lecce, Solicara	16. Aug. 2018	MP	*Messor ibericus*
17	Roma, Caffarella	16. May 2018	LL	*Messor ibericus*	43	Lecce, Solicara	16. Aug. 2018	MP	*Messor ibericus*
18	Roma, Caffarella	11. Jun. 2018	CML	*Messor ibericus*	44	Lecce, Solicara	Sept. 2018	MP	*Messor ibericus*
19	Roma, Caffarella	11. Jun. 2018	CML	---	45	Lecce, Solicara	Mar. 2019	MP	*Messor ibericus*
20	Roma, Malagrotta	09. Dec. 2018	LL	*Messor ibericus*	46	Lecce, Solicara	12. Apr. 2019	MP	*Messor ibericus*
21	Roma, Caffarella	20. Feb. 2019	LL	*Messor ibericus*	47	Lecce, Solicara	19. Apr. 2019	MP	---
22	Roma, Caffarella	15. Jun. 2019	LL	*Messor ibericus*	48	Lecce, Solicara	26. Apr. 2019	MP	*Messor ibericus*
23	Roma, Caffarella	23. Feb. 2020	LL	*Camponotus aethiops*	49	Lecce, Solicara	26. Apr. 2019	MP	*Messor ibericus*
24	Roma, Caffarella	23. Feb. 2020	LL	*Messor ibericus*	50	Lecce, Solicara	05. May 2019	MP	*Messor ibericus*
25	Roma, Caffarella	29. Sept. 2020	LL	*Messor ibericus*	51	Lecce, Solicara	05. May 2019	MP	*Messor ibericus*
26	Roma, Caffarella	21. Oct. 2020	LL	*Messor ibericus*	52	Buccheri (SR)	29. Sept. 2020	GR	---

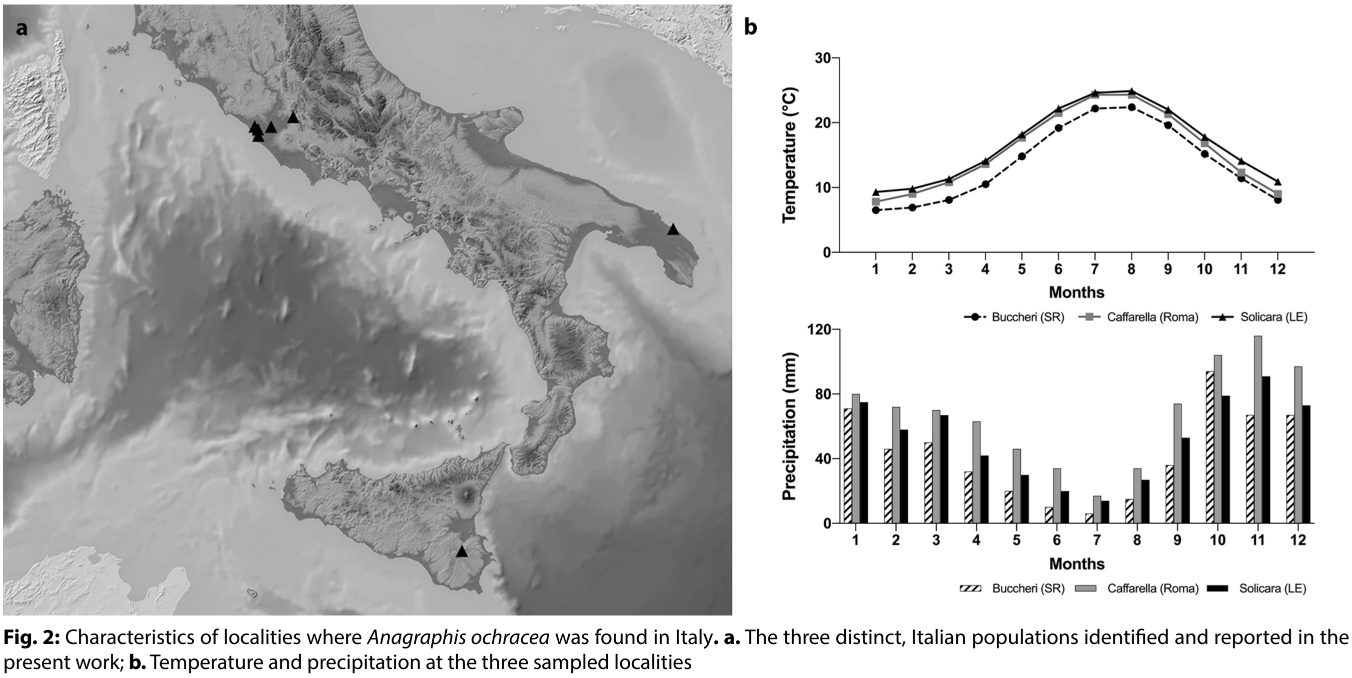

Fig. 2: Characteristics of localities where *Anagraphis ochracea* was found in Italy**. a.** The three distinct, Italian populations identified and reported in the present work; **b.** Temperature and precipitation at the three sampled localities

and a complete absence of a dorsal pattern. A detailed description of the species was provided by Chatzaki et al. (2002b). One distinctive feature that visually sets it apart from other Italian gnaphosids is the thick layering of setae covering the entire body. This confers to the spider a distinctive, velvety look. Hair and setae are abundant over the entire opisthosoma, especially in its frontal region, over all segments of legs and pedipalps and on the prosoma where, in the ocular region and on the chelicerae, long forward-projecting hairs can also be discerned (Fig. 1d).

No significant morphological differences were observed between the three Italian populations. Both genitalia and size

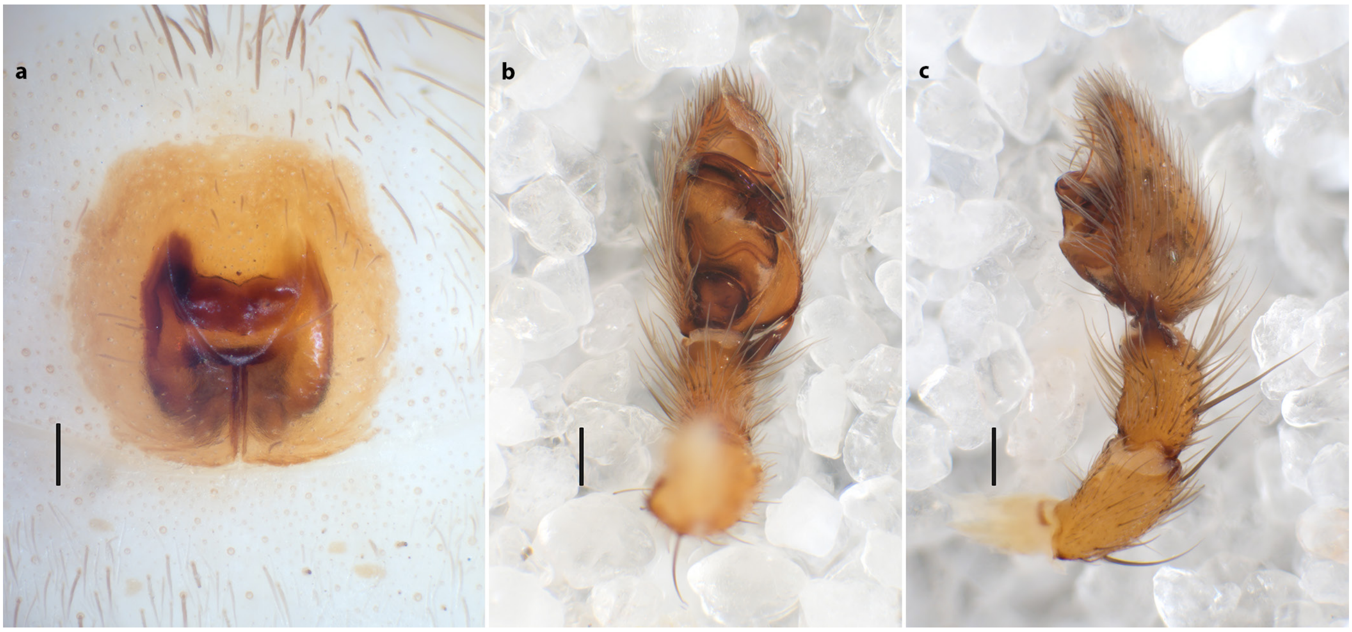

Fig. 3: *Anagraphis ochracea*. **a.** epigyne (scale bar: 0.1 mm); **b.** male left palp in ventral view; **c.** male left palp in retrolateral view (scale bar: 0.2 mm)

variability were found to be very consistent in adults, with body length ranging between 6-9 mm in females and 5-6.5 mm in males. The analysis of female epigynes and male palpal organs revealed little to no intraspecific variation between the three sampled localities (Fig. 3).

All Italian specimens were found to morphologically coincide with individuals of *A. ochracea* used for comparison, collected in Greece (Thessaly, Central Macedonia, Samos) by Dr. Maria Chatzaki (see list of materials). They also proved to be perfectly consistent with the holotype of *A. ochracea*. Conversely, and despite a superficial resemblance in general appearance, all Italian specimens were found to possess very different genitalia compared to individuals of the closely related *Anagraphis pallens* Simon, 1893. The latter were collected on the island of Karpathos and Rhodes and, once again, provided to us by Dr. Chatzaki.

Lastly, a morphological character worth mentioning that seems to have been ignored until now, is the presence of distinctly long, thin, often distally recurved setae on the dorsal side of most leg segments (Fig. 4).

DNA barcode

We have analyzed an *A. ochracea* specimen collected in Lecce, Solicara. Sanger sequencing produced a final sequence of 693 base pairs relative to a fragment of gene loci COI (accession number OP871103), which is the standard, DNA barcoding region used for metazoans, established by the International Barcode of Life Consortium (https://ibol.org). No frame shifts or stop codons were identified following translation of the nucleotide sequence to an amino acid sequence. The resulting DNA barcode is the very first to be made publicly available for both *A. ochracea* and the genus *Anagraphis*.

Ecological notes and behavioural observation in the wild populations in Lazio

The presence of *A. ochracea* in Italy and its myrmecophilic lifestyle were first recorded by Luigi Lenzini starting from November 2009. Specimens were found in the Appian Way Regional Park (Rome), more specifically in the section occupied by the Caffarella Park (20m a.s.l) (Fig. 2a). The Caffarella Valley preserves the typical scenery of the Roman countryside and is an integral portion of the vast Regional Park. Agropastoral activities, such as extensive agriculture and ovine breeding, survive to this day and coexist naturally with the inhabitants of the surrounding neighbourhoods that use the park for their own recreational activities. The valley is subject to a predominantly Mediterranean climate, and we report mean annual temperature and rainfall (Fig. 2b). The areas of

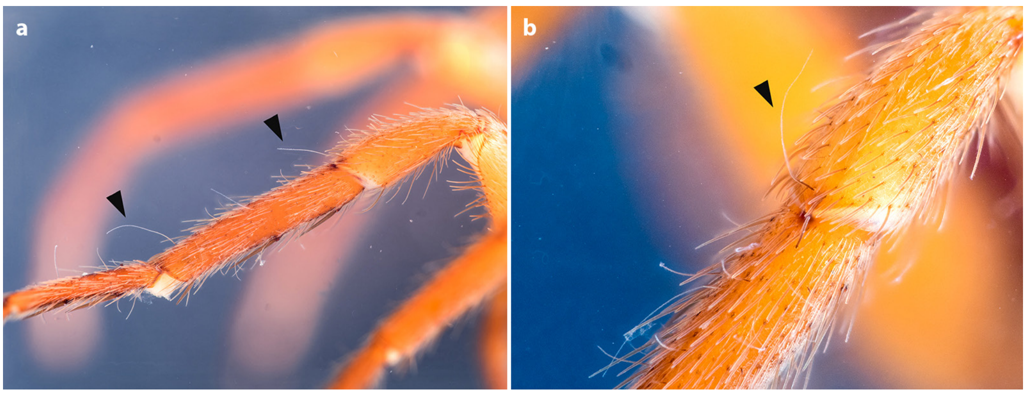

Fig. 4: *Anagraphis ochracea*, long setae on the dorsal side of legs. **a.** patella and tibia, **b.** close-up of long and distally recurved seta on joint of patella/tibia

the valley where specimens of *A. ochracea* were observed and collected are uncultivated pastures, grasslands and periodically mowed meadows, all with little to no tree cover.

The first two specimens observed were found after lifting a flat rock that covered a *Messor ibericus* ant colony. The area beneath the rock hosted several dozen worker ants and two spiders were observed moving among them without alarming the colony, never attempting to flee outside the area like most other spiders would do and never being attacked by the alarmed colony. Over the course of the following years, more than 30 similar observations were carried out in several other zones of the Caffarella Park (Tab. 1). Following more targeted searches, additional specimens were also found and observed in more distant localities around the city of Rome: in Ostia Antica (5m a.s.l) in December 2012, in Torrinpietra (60m a.s.l), Fiumicino, in October 2017, in Malagrotta (20m a.s.l) in December 2018 and October 2020 and, lastly, in Mount Catillo (330m a.s.l.), Tivoli, in April 2022. These additional locations differ little in habitat and vegetation cover to the areas of the Caffarella Park where the first specimens were observed. Exceptions include Ostia Antica, where the patch of land in which the specimen was found is used for horticulture and has higher average humidity levels, and Tivoli, a location of higher altitude compared to the rest, characterized by calcareous terrain and abundant rocky outcrops.

Over the course of 13 years, approximately 40 different, adult and juvenile specimens were observed and photographed in Rome and its immediate surroundings. Almost all individuals were found living amongst *Messor ibericus* colonies (Fig. 5). Only three instances involved different ant species, namely *Messor wasmanni* Krausse, 1910 in two occasions and *Camponotus aethiops* (Latreille, 1798) in one.

Specimens were not always found alone, and on several occasions, multiple *A. ochracea* were found living within the same colony, even at different developmental stages. In one instance, five different individuals were found beneath the same rock covering a *Messor ibericus* ant nest.

The dates in which specimens were observed or collected are essentially random and follow no regular pattern. They were, rather, directly affected by the seasonal activity of ants and by the periods of favourable conditions of the terrain. Most observations were, thus, carried out in spring and in autumn, when ant colonies are active, the vegetation of pastures and grasslands in the urban park is contained and the rocks that cover ant colonies are readily visible and easily accessible.

In most of the observations carried out in the Lazio region, *A. ochracea* was the sole arthropod species, besides the ants, found beneath the rock. Only on rare occasions were individuals found together with other myrmecophilous or soil-dwelling arthropods. These included orthopterans of the genus *Myrmecophilus*, several different isopod species (see Fig. 5) and centipedes of the genus *Lithobius*.

Population in Apulia

The number of observations and the unique behavioural nature of the findings in central Italy sparked interest and lead to targeted searches in other areas of the peninsula. Ultimately, between 2018 and 2019, *Anagraphis ochreacea* was found also in southern Italy, specifically in the region of Apulia, near Solicara, north-east of city of Lecce (Fig. 2a). Several specimens were observed and collected in a rural zone that featured an abundance of *Messor ibericus* colonies. This area is characterized by a dry Mediterranean steppe environment and is dominated by xerophilous herbs and grasses. Several plots of land have been dedicated to the cultivation of wheat and sunflower. The general lack of shrubbery, tree cover and shade mean that solar radiation is high and, accordingly, soil temperatures reach extreme levels during the summer season (Fig. 2b). The location where specimens were collected is a dry area bordered by a 200-meter-long rock mound covered in brambles and other vegetation. Here, numerous, large, well developed *Messor ibericus* colonies were found coexisting with several, smaller colonies of *M. wasmanni*.

In accordance with the observations carried out in central Italy, and except for a single wandering adult male, all specimens of *A. ochreacea* encountered in Apulia were found within or in immediate proximity to *Messor ibericus* ant nests. Most of these specimens were found dwelling unscathed within the superficial area of the colony beneath the rocks, surrounded by unalarmed *M. ibericus* workers. Several specimens, disturbed by the removal of the overlaying rock, were often observed quickly escaping and descending underground directly inside the colony, utilizing the busy entrance tunnels. On one occasion, ten small, juvenile specimens were found resting to-

Fig. 5: *Anagraphis ochracea*. The vast majority of the specimens were found together with *Messor ibericus* ants. **a.** female together with worker ants in Caffarella Park (Rome), **b.** ditto

gether inside a 15 cm deep chamber of the ant nest.

The frequency of the encounters in southern Italy was, for the most part, directly correlated with the periods of maximum activity of *M. ibericus*. As a result, most individuals were observed during spring and autumn when colonies reach peak activity levels, while very few were found in summer and winter, when surface activity of the ants is at its lowest. During the hottest months of the year, when maximum daily temperatures reach 38-39 °C, specimens of *A. ochreacea* were observed only during the twilight hours (between 19.30 and 20.00). This is when the ants abandon the protection of the thermally insulated depths and return to the superficial areas of the nest, directly underneath the rocks. All sampling and observations were carried out in dry conditions, never during rainfall or with wet terrain. Finally, in Apulia *A. ochreacea* was found coexisting on multiple occasions with soil dwelling isopods and, differently to what was observed in central Italy, myrmecophilous Zygentoma species.

Observations in Sicily

The specimen from Sicily was collected under drastically different environmental conditions. This single adult male, presumably wandering in the search of females, was found, surrounded by trapped dust, on the bathroom floor of a medical clinic. As a result, no observation on myrmecophilous behaviour and ecological information on the preferred natural habitat can be provided for the Sicilian population. However, the small town of Buccheri where the specimen was found, is located at 820 m a.s.l on the northern slope of Mount Lauro (986m a.s.l), in the province of Syracuse (Fig. 2a). The general area is characterized by a semi-continental, temperate climate. In the winter season, low clouds and foggy days are frequent and weak to moderate frosts can occur (Fig. 2b). The vegetation surrounding the town is mainly composed of young, Mediterranean, coniferous woodlands planted in the mid-1900s. At greater altitudes, around 920 m a.s.l, the trees give way to open, barren clearings mostly used for pasture.

Behavioural observations in controlled conditions

All specimens from Caffarella Valley observed in captivity proved to be highly adaptable to the captive space provided, showing no signs of erratic behaviour or stress. None ever attempted to escape the enclosure by climbing the slippery glass surfaces, nor did any ever try to dig a retreat in the substrate. Spiders generally stayed idle and remained motionless on the terrain, showing sudden bursts of movement only when disturbed or when prey items were dropped in the enclosure. Captive specimens were relatively long-lived, with a notable adult female still thriving after 19 months.

During captive breeding, several different prey items were offered to each spider to test the feeding habits of the species. *Messor ibericus* workers were entirely ignored on every occasion, and so were the larvae and pupae, suggesting that *A. ochracea* does not prey on ants. Isopods belonging to both Oniscidae and the Platyarthridae, common in large ant colonies, were also always neglected by the spider. On the other hand, all specimens proved to be extremely responsive towards several Zygentoma species (Lepismatidae - *Tricholepisma* sp.), as well as other more generic species such as the common *Thermobia domestica* Packard, 1873. Their presence always elicited a rapid reaction from the spider that promptly pounced on the prey and firmly grasped it with all its legs. None of the *A. ochracea* were ever observed utilizing silk during prey capture.

The level of predatory responsiveness shown towards lepismatids was quite remarkable when compared to other prey items. Interestingly, spiders aggressively hunted every lepismatid in the enclosure even when multiple specimens were dropped inside at the same time. The spiders promptly killed every specimen, and on many occasions did not even attempt to eat the multiple deceased prey.

Ten years of acquired experience of captive breeding have shown that *A. ochracea* is a spider that eats sporadically and can tolerate long periods of time without food. Hunted prey is often only partially consumed before being discarded. Lepismatid prey items were generally offered once every one or two months without ever observing a physical state of food deprivation in any of the spiders.

Two adult females built multiple egg sacs during captive breeding. The first laid eggs in the enclosure shortly after capture. The second female, captured on 8. Nov. 2020, laid four different egg sacs during captivity: one after 7 months (18. Jun. 2021), another on 1. Aug. 2021, a third egg sac after 12 months (21. Oct. 2021) and a final fourth after 18 months (31. May 2022). The egg sac of *A. ochracea* is circular, 5mm wide, dome-shaped, flattened on one side, whitish in colour (Fig. 6) and fastened to flat surfaces. On numerous occasions during field sampling, identical egg sacs were observed attached to the underside of the rocks that covered ant colonies known to host specimens of *A. ochracea*.

Fig. 6: *Anagraphis ochracea*. Adult female with egg sac and a small juvenile (arrow)

At least one of these egg sacs was fertile and hatched approximately one month after deposition (26. Aug. 2021). Though a tiny exit hole was observed on their surface, the hatching process was not immediately noticed as first instar juveniles are particularly small and can easily blend with the substrate. Only one of the hatched spiderlings was spotted in the enclosure (Fig. 6), so an average number of eggs laid by this species cannot be reliably provided.

An artificial ant nest of *Messor ibericus* was setup in an attempt to replicate, under visible lab conditions, the peculiar symbiosis observed in the wild between the two species. Due to the lack of any reliable scientific source in literature, no specific protocols or methodologies were followed during the setup of the formicarium. The authors attempted to ensure that the most optimal conditions were provided to guarantee reliable behavioural observations. A healthy and very populous, mature colony of approximately 2000 individuals was introduced in the artificial nest, and promptly settled in the overlaying arena and inside the numerous galleries and secondary chambers of the system.

Following the establishment of the colony, and during its most crowded phase, a single adult female of *A. ochracea* was introduced to the formicarium. After three full days of cohabitation, the spider was still unharmed and was observed calmly wandering around the main arena and the uppermost chambers, often walking directly on top of the ants (Fig. 7). The ants themselves appeared to tolerate its presence: workers would either briefly probe the spider with their antennae before continuing their work undisturbed or casually walk alongside it ignoring its presence entirely. On very rare occasions, some workers were observed trying to bite one of its legs, an attempt that regularly failed when the spider briefly sprinted off a short distance away. These sporadic attempts by individual ants never propagated into a collective alarmed or defensive state of the colony. They may, however, explain why on three separate occasions in the field, specimens were found to be either missing a limb or possessing a freshly regenerated appendage. Thus, it is plausible to assume that in certain rare instances, the spider may encounter brief acts of hostilities by its ant symbionts.

Several days later, a second adult female was introduced to the colony, once again in the arena of the formicarium. The same interspecific interactions were observed: the spider moved freely within the colony and the ants appeared to tolerate its presence entirely, carrying on with their regular activities undisturbed and unalarmed. This second female peacefully cohabited with the ants for one full day before being removed from the nest.

After having observed and confirmed a clear degree of mutual tolerance between *A. ochracea* and *M. ibericus* even in lab conditions, several other non-myrmecophilic species (*Steatoda nobilis, Lycosoides coarctata*, *Scotophaeus blackwalli* and *Filistata insidiatrix)* of spiders were introduced to test whether the same colony would react differently. Shortly after their introduction to the formicarium, each one of these species provoked clear, collective alarm and agitation, before being promptly attacked, overwhelmed and cut to pieces by the workers. This is not unusual as despite being primarily granivores, *M. ibericus* will occasionally feed on live or dead invertebrates such as earthworms, insect larvae and other arthropods as well as defend its nest voraciously against intruders.

Myrmecophily of *Anagraphis ochracea*

Numerous, repeated observations carried out in the wild in both Lazio and Apulia, clearly demonstrate the remarkable habit of *A. ochracea* to live, undisturbed, within ant colonies. Except for three isolated cases that involved *Messor wasmanni* and *Camponotus aethiops*, the majority of the specimens were found in association with *Messor ibericus.* Interestingly, when disturbed by the removal of the overlying rock, rather than running away from the alarmed ants like most other spider species do, *A. ochracea* always remained among the startled colony and often escaped directly inside the busy entrance tunnels of the nest. Additional lab observations unequivocally confirmed that *A. ochracea* is capable of blending within and being accepted even by a newly encountered ant colony. This is in stark contrast to what was observed with other spider species instead, all of which were promptly overwhelmed by workers and disposed of shortly after their introduction to the formicarium.

Ant colonies can be considered complex ecosystems on their own. Each ant fulfils a specific role to help the colony survive. Defence and protection are of utmost importance: the worker caste will staunchly defend the colony from potential predators, and any external intruder is generally considered a threat (Hölldobler & Wilson 1990). Nevertheless, a great number of arthropod species, both arachnid and insect, have developed ways to live unharmed within the colonies, in a symbiotic relationship with ants. Ant nests, in fact, provide a significant number of ecological benefits to would-be hosts, including a relatively stable microclimate, a constant abundance of food sources and a considerable level of protection from their own natural predators (Cushing 1997, 2012, Witte et al. 2008, Nelson & Jackson 2009). Evidently, for such a risky evolutionary adaption to be successful, the benefits must

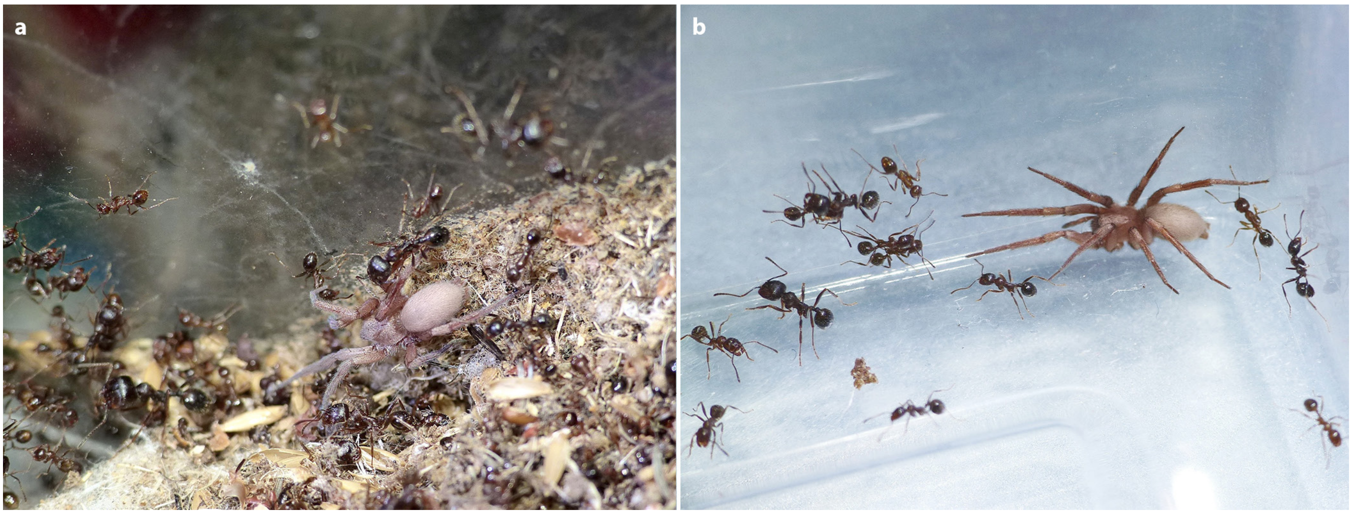

Fig.7: *Anagraphis ochracea*. The female specimens used in the experiment moved freely among workers in the arena of the artificial formicarium. **a.** adult female surrounded by unalarmed *Messor ibericus* worker, **b**. ditto, in a debris-free area of the arena

greatly outweigh the drawbacks of living inside a thriving ant colony (Ceccarelli 2013). To establish a stable symbiotic relationship with ants, these arthropods, generically known as myrmecophiles, have developed ways of penetrating the biological barriers of the colony in order to survive unharmed or unseen. The means through which myrmecophiles manage to blend in such a risky environment are varied and can differ substantially between species, and may be of morphological, behavioural, and chemical nature (Cushing 1997, 2012).

The observational scope of this study did not allow us to confidently affirm through which mechanism *A. ochracea* successfully manages to survive unscathed within *M. ibericus* colonies. Whether it be through mechanical or chemical processes is unclear, and further, more thorough analysis on cuticular hydrocarbons of both species will be carried out in the future.

Anagraphis ochracea is not a myrmecomorph, and lab observations indicate that it is in no way myrmecophagous either. Thus, it is easy to assume that it utilizes *Messor ibericus* colonies to find refuge, protection from external threats and easy access to a wide range of varied, potential food sources. In fact, observations in the field have revealed that Italian *M. ibericus* colonies harbour a wide range of different myrmecophilous arthropods. We recorded the following:

- Isopoda: Porcellionidae and Platyarthridae;
- Zygentoma: Lepismatidae (Tricholepisma and Neoasterolepisma);
- Orthoptera: *Myrmecophilus*;
- Chilopoda: Lithobiidae (*Lithobius romanus* (Marzio Zapparoli pers. comm.))
- Coleoptera: *Oochrotus* e *Cholovocera*;
- Acari: Laelapidae and Scutacaridae (Massimo Plumari pers. comm.)
- Collembola: Cyphoderidae

With the exception of lithobid centipedes, powerful predators on their own, and the minute sized Acari and Collemboli, which could possibly serve as prey items for the newly hatched spiderlings, the more likely food source for adult *A. ochracea* include myrmecophilous Zygentoma and Orthoptera.

Testing in captivity showed that when introduced to myrmecophilous lepismatids, *A. ochracea* always reacted very aggressively, promptly subduing the prey even when multiple Zygentoma were offered at the same time. No experiments were carried out utilizing orthopterans of the genus *Myrmecophilus*.

Years of experience in the captive breeding of this species have indicated that *A. ochracea* is not a voracious feeder and possibly possesses a slower metabolism compared to other Gnaphosidae. It appears to be a slow-growing species capable of living multiple years. The fact that both small juveniles and adults of both sexes were observed living inside healthy, thriving *M. ibericus* colonies leads us to believe that *A. ochracea* likely completes its entire life cycle in symbiosis with ants. Observations carried out in the lab also suggest that adult females lay multiple eggsacs and can preserve male sperm for a several months after mating.

Conclusions

The present work provides the first records of *Anagraphis ochracea* for both continental Italy and the island of Sicily, with two distinct populations found in the former. Observations in the field and experimentation in captivity unequivocally confirm that this species has a myrmecophilous lifestyle and lives as a symbiont inside colonies of *Messor ibericus*. In the wild, specimens of *A. ochracea* were commonly observed calmly resting even while surrounded by dozens of alerted worker ants and were often seen escaping directly inside the crowded tunnels of the alarmed colony. The use of an artificial formicarium of *M. ibericus* further confirmed that, as opposed to other spider species tested, *A. ochracea* is readily accepted by the ants and will survive unscathed for days. In conclusion, this species can live undisturbed and unalarmed inside *M. ibericus* colonies and does so possibly using some form of chemical mimicry.

As a result, we demonstrate for the very first time that, at least in the areas we studied, this species lives as a myrmecophile inside ant colonies. However, we cannot yet determine and describe which mechanism is in play during this symbiosis, be it mechanical, chemical or of other nature. Further research on the cuticular hydrocarbons of both symbionts will be carried out by the authors. Ultimately, the present work shows how a targeted sampling of obscure, overlooked, hardly accessible and often neglected microhabitats, such as an ant colony, can lead to interesting new discoveries from a biogeographical, behavioural, and ecological point of view.

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