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ORIGINAL PAPER

Insects inhabiting fruiting bodies of Burgundy truffle *Tuber aestivum* Vittad. in Poland

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ABSTRACT

The aim of this study was to investigate the species composition of insects inhabiting the fruiting bodies of Burgundy truffle *Tuber aestivum* in Polish forests. Field work was carried out in 2016-2017 at four natural truffle sites in southern Poland. The fruiting bodies were searched with the help of a trained truffle dog or collected randomly. Adult insects were captured directly from the fruiting bodies using an exhaustor. Fruit bodies that were possibly inhabited by insect larvae were collected, brought to the laboratory and placed in special containers to develop them. Insects were reared in a biological chamber with constant parameters. Adult insects collected from the field and reared in the laboratory were identified using morphological methods. During the study, 584 Burgundy truffle sporocarps were found and examined (364 in 2016 and 220 in 2017). In 2016, 90 truffles were infested by insects (about 24.7%), while in 2017, 93 fruiting bodies were damaged, representing 42.2% of the harvest. A total of 330 insect specimens belonging to 21 species were identified. Some of them can cause significant losses in truffle plantations. Others can be considered as indicators of the presence of truffles in the forest environment. During the study, 236 specimens of Diptera were obtained from breeding, and a total of 86 imagines of Coleoptera and 8 imagines of Hymenoptera were collected directly from the fruiting bodies. Ant larvae were also observed in the truffles but were not collected for breeding. The assemblage of insects inhabiting truffles was identified, with two visibly dominant

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species: *Cheilosia soror* fly from the Syrphidae family and truffle beetle *Colenis immunda* from Leiodidae family. Among other common insect species, the flies *Suillia affinis* and *Suillia pallida* and the beetles *Leiodes cinnamomea* and *Leiodes oblonga* were observed. For the first time, a case of colonization of truffle fruiting bodies by ants *Myrmica rubra* has been described. The pioneering research carried out in Polish forests has helped to significantly increase knowledge about truffle-inhabiting insects, their occurrence and biology, which is necessary to reduce the damage they cause to truffle production.

KEY WORDS

Heleomyzidae, Leiodidae, Staphylinidae, Syrphidae, truffle cultivation, truffle pests

Introduction

Truffles *Tuber* spp. are fungi with underground fruiting bodies that grow exclusively outdoors, in different types of wild plant formations around the world (usually in forests and thickets) (García-Montero *et al.*, 2007; Garcia-Barreda and Reyna, 2012; Molinier *et al.*, 2016; Hilszczańska *et al.*, 2019; Mleczko *et al.*, 2023) or in special agroforestry systems (truffle orchards/plantations) managed by humans (Bruhn and Hall, 2011; Benucci *et al.*, 2012; Byé, 2020; Hilszczańska *et al.*, 2022). In any case, the truffle life cycle, despite many uncertainties, follows the rhythm of nature (Payen *et al.*, 2014; Le Tacon *et al.*, 2016). The cultivation of truffles is a special agroindustry with great potential (Zambonelli *et al.*, 2015; Sourzat, 2020). The fruiting bodies are extremely valuable, but due to the specificity of cultivation, the investment is associated with certain risks (Stobbe *et al.*, 2013). The growth of the host trees and the development of the fruiting bodies are influenced by atmospheric conditions: temperature, precipitation, solar radiation, number of frost days and soil (chemical and granulometric composition of the soil, *etc.*) (Wedén, 2009; Bruhn and Hall, 2011; Csorbainé, 2011; Stobbe *et al.*, 2013; Hilszczańska *et al.*, 2019; Rosa-Gruszecka *et al.*, 2019). Truffle plantations have been established, especially in Mediterranean countries where the truffle occurs naturally, where the precious fungus is an iconic product of culinary culture (Aumeeruddy-Thomas *et al.*, 2016) and recently also in many non-European countries in both hemispheres (Leonardi *et al.*, 2021). However, in less truffle-friendly areas, the deficiencies can be compensated by various treatments. For example, it is possible to maintain the proper level of the soil water retention (pF) curve by irrigating at appropriate times (Todesco *et al.*, 2019). In regions where the sun is too strong, shade mats can be used. Many processes can be influenced and stimulated, but still truffle orchards are exposed to two types of problems: first, sudden and unusual weather phenomena, *e.g.* heat waves, floods, tornadoes, which are part of the effects of climate change and whose occurrence we cannot control (Baragatti *et al.*, 2019; Thomas and Büntgen, 2019; Čejka *et al.*, 2022; Steidinger *et al.*, 2022). Second, the development of diseases on the fruiting bodies of truffles, caused mainly by insects and to a lesser extent by fungi and bacteria. In the first case, we can do little and concentrate more on eliminating the negative effects of the natural disturbances, *e.g.* by irrigating the truffle crops during the drought. The situation is different if the truffle crops are threatened by pests. Precise preventive measures are possible here, provided that the truffle pests have been identified.

The hypogeous development of the fungus makes it impossible for it to spread its spores with air or water currents (Ori *et al.*, 2021). The spread of truffle spores is based on the relationship with animals that eat fruiting bodies attracted by their aromas (Ori *et al.*, 2018; Thomas and Thomas, 2022). The aroma of truffles is very complex, it contains many components, not all of which are known. The aroma depends not only on the species of truffle, but also on other factors, such as the degree of maturity of the truffle and the composition of the microbiota that inhabit the surface and interior of the fruiting body (Pacioni *et al.*, 1991; Vahdatzadeh *et al.*, 2015). Substances such as dimethyl sulfide (DMS) and dimethyl disulfide (DMDS) (Allen and Bennett, 2021) attract not only large and small mammals such as wild boars, porcupines, and voles, but also numerous species of mycophagous insects. Some species not only eat the fungus, but also lay their eggs in it. In this context, beetles from the family Leiodidae, and flies of the genus *Suillia*, whose larvae develop in the fruiting bodies and damage these valuable fungi, should be mentioned in particular. The larvae not only eat the flesh of the fungus, but also accelerate its rotting (Di Santo, 2013) and damage it (Sitta and Süss, 2012). Although they are considered pests in truffle cultivation, in nature the insects are an important part of the truffle life cycle (Elkhateeb and Daba, 2021). Truffle spore dispersal can occur in two ways. Spores can be transmitted on the outside of the insect (epizoochory) or by ingestion and excretion from invertebrates (endozoochory).

Current knowledge about the insects inhabiting truffles is very incomplete, partly due to the underground development of these fungi and the hidden development of many insect species associated with them. So far, thanks to the research of the Forest Research Institute, the relationship of *Suillia pallida* (Fallén, 1820) and *Leiodes oblonga* (Erichson, 1845) with *Tuber* spp. has been discovered, among others (Rosa-Gruszecka *et al.*, 2017). So far, molecular methods of identification were only used for one individual of *Suillia gigantea* (Meigen, 1830) (= *Helomyza tuberivora*) (Laboulbène and Mignaux, 1864) (Rosa-Gruszecka *et al.*, 2023a). The other collected material needs further studies. Several species are mentioned in the literature in connection with truffles. *Leiodes cinnamomea* (Panzer, 1793) is considered to be the main cause of damage to fruiting bodies of valuable truffle species, including *Tuber melanosporum* Vittad. and *Tuber magnatum* Picco (Hochberg *et al.*, 2003). In Spain, the fruiting bodies of *T. melanosporum* are additionally damaged by *S. gigantea*, *Drosophila funebris* (Fabricius, 1787), *Ommatoiulus sabulosus* (Linnaeus, 1758) and representatives of the genera *Pleurophorus* Mulsant, 1842, and *Megaselia* Rondani, 1856 (Martín-Santafé *et al.*, 2014).

Despite more than 50 years of research on this topic, new questions continue to arise. What is certain is that the relationship between fungi and insects is extremely complex. Therefore, the aim of our study was to gain new insights into the insects associated with Burgundy truffle and to learn new details about their ecology in Poland.

Materials and methods

The field works were carried out in 2016-2017 on four natural truffle' sites located in two macroregions (Nida Basin and Przedbórz Upland) in the southern Poland. The studied sites favour the occurrence of many species of truffles such as *Tuber aestivum* Vittad., *Tuber excavatum* Vittad., *Tuber fulgens* Quél, *Tuber macrosporum* Vittad., *Tuber maculatum* Vittad. and *Tuber rufum* Picco. They are found on calcareous rocks: marlstone, gypsum or limestone, between 225 and 325 m above sea level. The vegetation (forest or thicket) is characterized by a predominance of deciduous tree or shrub species, including *Acer campestre* L., *Acer pseudoplatanus* L., *Carpinus betulus* L., *Corylus avellana* L., *Fagus sylvatica* L., *Quercus petraea* (Matt.) Liebl., *Quercus robur* L., *Populus tremula* L., *Prunus avium* L. and *Tilia cordata* Mill. (Hilszczańska *et al.*, 2019). Most of them are species that form a mycorrhizal symbiosis with truffles.

The fruiting bodies were searched with the help of a trained truffle dog of the Lagotto Romagnolo breed or randomly collected. Each year, 5 controls were carried out at each site from August to October (on average every 2 weeks). Study data were obtained by two methods for collecting insects associated with truffles.

First, adult insects were collected directly from the fruiting bodies of Burgundy truffle (*T. aestivum*) during truffle harvest at natural sites. All adult specimens were then captured using exhaustor and preserved in 2 ml Eppendorfs containing 70% ethanol.

Further, the collected truffle fruiting bodies were examined for the presence of insect larvae. The fruiting bodies inhabited by larvae were packed separately in cloth pouches and brought to the laboratory in a portable refrigerator. Each fruiting body was carefully cleaned of soil debris, weighed, and placed separately in a ventilated container (1.5 litres).

The fruiting bodies in the container were covered with a layer of rendzic soil (approximately 250 g) from one of the sites studied to ensure proper moisture and thermal conditions for the developing larvae. Previously, the soil was sterilized twice at 121°C for 20 min and sieved through a sieve (2 mm mesh) to remove soil insects, myriapods, nematodes, *etc*. Then, rearing was performed in the laboratory in a Mytron-type biological chamber (temperature about 13°C, humidity ~80%). Soil humidity was maintained at 15%. When fruiting bodies were eaten, larvae were additionally fed with frozen preserved fruiting bodies (which were not inhabited by larvae). Breeding was monitored for 2 years and checked every 10-14 days.

Adult insects collected from the field and reared in the laboratory were marked with an identification number and identified by morphological methods. The material was kept in plastic vials using pure 70% ethanol. The total specimens were examined under a Nikon SMZ 800 stereomicroscope, and the male and female terminalia were cleared in a solution of 10% NaOH, and studied under a ZEISS Primo Star light microscope, and finally placed in a microvial with glycerine. All specimens were identified using specific keys:

Hymenoptera:
– Formicidae Latreille, 1809 (Czechowski *et al.*, 2012);

Coleoptera:
– Carabidae Latreille, 1802 (Pawłowski, 1974),
– Geotrupidae Latreille, 1802 (Stebnicka, 1976),
– Leiodidae Fleming, 1821 (Daffner, 1982; Nunberg, 1987),
– Staphylinidae Latreille, 1802 (Szujecki, 1961);

Diptera:
– Drosophilidae Rondani, 1856 (Bächli *et al.*, 2004),
– Heleomyzidae Westwood, 1840 (Gorodkov, 1989),
– Mycetophilidae Newman, 1834 (Hutson *et al.*, 1980),
– Sciaridae Billberg, 1820 (Søli *et al.*, 2000),
– Sphaeroceridae Macquart, 1835 (Roháček, 1998),
– Syrphidae Latreille, 1802 (Van Veen, 2004).

The dominance structure was determined separately for 2 groups: adult beetles and hymenopterans caught in the field (F), and for flies bred from larvae in the laboratory (L). The dominance (D%) shows the participation percentage of each species in the group. Explains the relationship of a species herd with the sum of the individuals of the other associated species according to criteria given below.

This indicator is calculated according to the equation:

$$D_A = (N_A \cdot 100) / N_1$$

where:

D – dominance,

A – species abundance,

N_A – the total number of individuals of A species,
N_1 – the total number of individuals of the collected species.

Dominance classes include species whose spread percentage falls within the following values (Tischler, 1949):

D_1 – subrecedent species P<1.0%,
D_2 – recedent species P=1.1-2.0%,
D_3 – subdominant species P=2.1-5.0%,
D_4 – dominant species P=5.1-10.0%,
D_5 – eudominant species P>10.1%.

Results

During the study, a total of 584 fruiting bodies of *T. aestivum* were collected (364 ascomata and 220 in 2016 and 2017, respectively). In 2016, 90 truffles were infested by insects (about 24.7%), while in 2017, 93 fruiting bodies were damaged, representing 42.2% of the harvest. The mean weight of the collected truffles was 23.66 g, while the colonized fruiting bodies weighed 25.30 g on average. Undamaged truffles were found in only 1 out of 10 field controls (end of September 2017).

A total of 330 insect specimens were collected: 170 in 2016 and 160 specimens 2017 (Table 1). During the study, 236 specimens of Diptera were obtained from breeding, and a total of 86 imagines of Coleoptera and 8 imagines of Hymenoptera were collected directly from the fruiting bodies (Table 1). Ant larvae were also observed in the truffles but were not collected for breeding. The insects collected directly from the truffle fruiting bodies in the field belonged to 13 species. Seven insect species were observed in both 2016 and 2017, namely the flies *Cheilosia soror*

Table 1.
Insects collected and reared from Burgundy truffle *Tuber aestivum* Vittad. in Poland in 2016-2017 (* specimens collected as imagines from truffles)

Order	Family	Genus	Species	Number of specimens
Hymenoptera	Formicidae	*Myrmica*	*Myrmica rubra**	7
		Stenamma	*Stenamma debile**	1
Coleoptera	Carabidae	*Trechus*	*Trechus quadristriatus**	1
	Geotrupidae	*Trypocopris*	*Trypocopris vernalis**	1
	Leiodidae	*Colenis*	*Colenis immunda**	37
		Leiodes	*Leiodes cinnamomea**	12
			*Leiodes oblonga**	14
		Ptomaphagus	*Ptomaphagus sericatus**	2
	Staphylinidae	*Atheta*	*Atheta crassicornis**	7
			*Atheta gagatina**	6
		Gyrohypnus	*Gyrohypnus punctulatus**	1
		Neohilara	*Neohilara subterranea**	4
		Tachinus	*Tachinus laticollis**	1
Diptera	Drosophilidae	*Drosophila*	*Drosophila immigrans*	3
	Heleomyzidae	*Suillia*	*Suillia affinis*	14
			Suillia gigantea	2
			Suillia pallida	6
	Mycetophilidae	*Leia*	*Leia bimaculata*	12
	Sciaridae	*Sciara*	*Sciara* sp.	1
	Sphaeroceridae	Limosininae	Limosininae sp.	1
	Syrphidae	*Cheilosia*	*Cheilosia soror*	197

(Zetterstedt, 1843) (=*Ch. rufipes* Preyssler, 1798), *Suillia affinis* (Meigen, 1830) and *S. pallida* as well as the beetles *Colenis immunda* (Sturm, 1807), *Leiodes cinnamomea*, *L. oblonga*, and the rove beetle *Atheta gagatina* (Baudi di Selve, 1848).

Ants belonging to two ant species were observed on the fruiting bodies of the subterranean fungi: *Myrmica rubra* (Linnaeus, 1758) (seven specimens) and *Stenamma debile* (Foerster, 1850) (one individual). In the case of *M. rubra*, all ants were found in the cava of the fruiting body of the Burgundy truffle, where they had their nest. We observed both larvae and imagines. The beetles were represented in large numbers. The individuals captured from fruiting bodies belonged to four families: Carabidae, Geotrupidae, Leiodidae and Staphylinidae. The family Leiodidae was the most numerous with 66 individuals belonging to four species. It was dominated by the species of beetle *C. immunda* (37 specimens). *Leiodes oblonga* (14 specimens) was slightly more numerous than *L. cinnamomea* (12 specimens). In September 2017, one male and one female of *Ptomaphagus sericatus* Chaudoir, 1845 were captured at one of the sites in the Nida Basin. The family Geotrupidae was represented by one specimen of *Trypocopris vernalis* (Linnaeus, 1758) and the family Carabidae by *Trechus quadristriatus* (Schrank von Paula, 1781). The family Staphylinidae was more numerically represented with individuals of five species: *Atheta crassicornis* (Fabricius, 1793), *A. gagatina*, *Gyrohypnus punctulatus* (Paykull, 1789), *Neohilara subterranea* (Mulsant *et* Rey, 1853) and *Tachinus laticollis* Gravenhorst, 1802. All beetles are imagines collected in the field from truffle fruiting bodies. In contrast, the representatives of Diptera were only from reared larvae. Adult insects from six families were reared. The dominant species (197 individuals) was the *Ch. soror* fly (Table 2).

Table 2.
Dominance structure of insects inhabiting Burgundy truffle fruiting bodies *Tuber aestivum* Vittad. in Poland in 2016-2017

Species	Insect group (F – field, L – laboratory bred)	Number of individuals	Dominance rate [%]	Dominance class (P)
Colenis immunda	F	37	39.4	eudominant
Leiodes oblonga	F	14	14.9	eudominant
Leiodes cinnamomea	F	12	12.8	eudominant
Atheta crassicornis	F	7	7.4	dominant
Myrmica rubra	F	7	7.4	dominant
Atheta gagatina	F	6	6.4	dominant
Neohilara subterranea	F	4	4.3	subdominant
Ptomaphagus sericatus	F	2	2.1	subdominant
Gyrohypnus punctulatus	F	1	1.1	recedent
Stenamma debile	F	1	1.1	recedent
Tachinus laticollis	F	1	1.1	recedent
Trypocopris vernalis	F	1	1.1	recedent
Trechus quadristriatus	F	1	1.1	recedent
Cheilosia soror	L	196	83.1	eudominant
Suillia affinis	L	14	5.9	dominant
Leia bimaculata	L	12	5.1	dominant
Suillia pallida	L	6	2.5	subdominant
Drosophila immigrans	L	3	1.3	recedent
Suillia gigantea	L	2	0.8	subrecedent
Limosininae sp.	L	1	0.4	subrecedent
Syrphidae sp.	L	1	0.4	subrecedent
Sciara sp.	L	1	0.4	subrecedent

There are 3 species of ‘truffle flies’ of the genus *Suillia*: *S. affinis*, *S. gigantea* and *S. pallida*. The fungus gnat *Leia bimaculata* (Meigen, 1804) was reared from three fruiting bodies. Single specimens of flies from the genera *Drosophila* Fallén, 1823, *Sciara* Meigen, 1803, and a member of the subfamily Limosininae Frey, 1921, were also reported.

Discussion

Knowledge of the insects that inhabit truffle fruiting bodies is important in two aspects. First, invertebrates play a key role in the dispersal of truffle spores in nature. These interactions are still very poorly understood, but at the same time of great importance from a biological point of view (Menta and Pinto, 2016). This project contributes to a better understanding of the relationships in forest ecosystems where fruiting bodies of underground fungi occur. It has increased knowledge about valuable fungi and numerous insect species, many of which are considered rare. The second aspect is the protection of truffle crops. According to the available studies, the main problems in truffle cultivation today are caused by truffle beetles (*L. cinnamomea*) and truffle flies (*S. gigantea*) (Martín-Santafé *et al.*, 2014). However, our studies show that the most numerous species could be other, rarely mentioned insects, such as the beetle *C. immunda* and the fly *Ch. soror*. Identifying the insects that inhabit truffles is the first necessary step in protecting these valuable crops. This is particularly important because in this study the estimated percentage of truffle colonization by insects is very high (24.7 and 42.2%, depending on the year). Without knowledge of the insect species threatening the crops, there is no way to control them, *e.g.* by using selective pheromone traps (De Bree, 2022; Taschen *et al.*, 2022; Rosa-Gruszecka *et al.*, 2023a). This study provides new data on insects associated with truffles, both as potential pests and as rare species for which new evidence is presented.

The main resources that animals, including insects, need to maintain their metabolism and complete their life cycle, *i.e.* to grow and reproduce, are nutrients (food), water and a habitat that supports the survival and reproduction of a particular species (Wu, 2022). Each habitat consists of a large number of microhabitats that differ in terms of light, humidity, temperature, air movement and other factors (Cloudsley-Thompson, 1962). In a forest, there are many different microhabitats (*e.g.* rotten wood, buttress roots, fallen tree trunks, grass tussocks, fungi) inhabited by microfauna, *i.e.* countless species of invertebrates, each with their own specific habitat requirements (Packham *et al.*, 1993; Vítková *et al.*, 2018). The life cycle of some insect species includes several, sometimes very different, microhabitats, depending on their life stage (Kingsolver *et al.*, 2011). The truffle fruiting body is a relatively rare and specific microhabitat that fulfils the small-scale physical requirements of a particular organism or population. The fruiting body of a truffle can fulfil numerous functions for different insect species that are more or less closely related to it. Certain insects only hunt on the surface of the truffle (Staphylinidae family), others just feed on the fruiting body, *e.g. Trypocopris vernalis* (Linnaeus, 1758). According to the work of Pacioni *et al.* (1991), numerous Staphylinidae species can be associated with truffles, but none of the 19 species mentioned in the Italian study were found in this study. Only a single ground beetle, *T. quadristriatus*, was caught on the fruiting body and was possibly hunting in the vicinity of the fruiting body. The truffle as an environmental resource can serve as a shelter for some of the invertebrates. For example, the basal cavity of the fruiting body can also be used by ants to build nests, as was the case in this study. At the same time, the inside of the fruiting body remained uninhabited by other insects. We agree with the hypothesis (Pacioni *et al.*, 1991) that ants could feed on the larvae inside the truffle. In this case, the ants (*M. rubra*) may have effectively prevented other insects from colonizing the fruiting body. Especially that *M. rubra* is a stinging species and has been suggested to be the most aggressive of the *Myrmica* species in Europe (Elmes, 1991). Most importantly, the strongest bond between insects and truffles is with those whose larvae develop inside the fungus, especially the representants of families Leiodidae, Heleomyzidae, Mycetophilidae and Syrphidae. Beetles belonging to Leiodidae are more likely to attack the fruiting bodies of *T. melanosporum*, while those of *T. magnatum* are more likely to be attacked by larvae of Diptera (Sitta and Süss, 2012).

The coleopteran fauna associated with truffles in literature is mainly represented by the beetle *L. cinnamomea*. Adult females of this species are attracted to truffles at an early growth stage (Hochberg *et al.*, 2003). In the present study, three eudominant species from the family Leiodidae were identified, of which *L. cinnamomea* was the least common. The morphologically very similar *L. oblonga* was reported slightly more frequently. Surprisingly, the small truffle beetle *C. immunda* was encountered most frequently.

The fruiting bodies of truffles are very frequently attacked by various *Suillia* species (Ciampolini and Süss, 1982; Krivosheina, 2008), whose adult females fly close to the surface and lay their eggs on the ground above the truffle fruiting bodies so that the larvae can easily reach them when they hatch. Chandler (2010) reports that Diptera of the genus *Cheilosia* (Syrphidae) are also associated with *Tuber* species. Concerning the Diptera species found in Burgundy truffle, two species can be considered as dominant. There are: the eudominant hoverfly *Ch. soror*, and the dominant heleomyzid fly *S. affinis*. The majority of *Suillia* larvae are subservient to mushrooms, some of them especially truffles. All recorded herein *Suillia* species are known as pests of European truffles (Rosa-Gruszecka *et al.*, 2023b). Among them, *S. affinis* is the most common heleomyzid species recorded in the central part of the Middle-Eastern Europe. However, *S. pallida* is the most frequent truffle fly known from Scandinavia, especially Sweden (Struwe and Wedén, 2016). Both species are often recorded in Europe as overwintering imagines (Soszyńska and Woźnica, 2016), but the biology of the larval stages has not been thoroughly investigated. It appears that the larvae can develop underground in the winter, in the fruiting bodies of subterranean fungi. The larvae of *Ch. soror* feed in the stems of plants or in fungi. The adults of *Ch. soror* fly from May to September, with a peak in June/July. Its larva was reported as having been found in truffles (Ballester-Torres *et al.*, 2022). This fact was finally confirmed immature stages found in the fruiting bodies of burgundy truffle by Rosa-Gruszecka *et al.* (2023b).

Among all known vinegar flies, *Drosophila immigrans* Sturtevant, 1921, a species native to Asia, and currently known as common vinegar species fly with red eyes, has been associated with various fungi, also with fruit and even garden compost heaps. It has been reported that the male's method of mounting and grasping the female during copulation is peculiar and very rare observed in the genus *Drosophila*, and the male possesses at the inner margin of the femur a row of stout, peg-like setae and shortened first tarsal segments which also bear heavy setae (Bächli *et al.*, 2004), and uses such specialized forelegs to aid in grasping the female (Spieth, 1952). Such chaetotaxy and copulation is known in *S. gigantea* (Rosa-Gruszecka *et al.*, 2023a). Contrary to the golden truffle fly, *D. immigrans* is an eurythermal species with a physiological temperature adaptation to warm and cool environments (Hunter, 1968) therefore not strictly connected under limited climate condition (*i.e.* global warming processes), and can be with all probability expected as a potential truffles pest in many European countries. So far it has not been detected in truffles. This is the first record of imagines of *D. immigrans* reared from *T. aestivum*.

Concerning the Mycetophilidae reared from the black truffle, only one species was found – *Leia bimaculata*. Within the genus *Leia* Meigen, 1818, nineteen species occur in Europe; but rearing records exist for eight of them only. Generally the larvae live in a slimy web on the under surface of fungi, or on the surface or under bark of rotting wood (Jakovlev, 2011). So far, imagines of *L. bimaculata* has been only reared from Basidiomycota, from more ca 20 genera of fungi especially Agaricomycetes (Dely-Draskovits and Babos, 1993). In Poland was recorded from *Russula delica* Fr. in the Nida Valley (Mikołajczyk, 1967).

The other two taxa of flies are represented only by single females, the *Sciara* sp., and the dung fly of the subfamily Limosininae (genus indet.) can be classified only as subrecedent taxa. Concerning the known biology of Limosininae species, most larvae are rather saprophagous and related to decaying plants, dung, or feed on decomposing fungus tissue (rotten fungi) rather (Roháček and Papp, 2021) than are typical pests of fruiting mushrooms.

Whereas the dark-winged fungus gnats (Sciaridae) are sometimes well-known pests of mushrooms and are often sampled on the fruiting body in greenhouses (*i.e. Lycorellia* spp. and *Bradysia* spp.), the *Sciara* species identified only to the genus level is probably not a true fungus feeder. The larvae of most Sciaridae are rather typical soil inhabitants, where they feed on various decaying vegetations (Ševčík, 2010). Krivosheina (2008) noted that further research is needed to clarify if they really feed on the living tissues of the mushrooms fruit bodies, but Hall and Gerhard (2002) report that *Lycoriella mali* (Fitch, 1856) is a major pest of commercial mushrooms, feeding on all stages of fungi, and *Bradysia* species are known to infest greenhouses, where they *i.e.* consume fungi in potting soil; transmitting spores of plant-parasitic fungi of the genus *Pythium* Pringsh.

Another interesting observation concerns the relationship between ants and truffles, which has not yet been scientifically investigated. *M. rubra* is one of the most widespread *Myrmica* species of Palearctic north temperate ant species with a native range that extends from Ireland and Great Britain, through northern Europe to western Siberia (Czechowski *et al.*, 2012). It is very common in Europe and is considered now invasive in some parts of North America and Asia (Groden *et al.*, 2005; Czechowski *et al.*, 2012; Seifert, 2018). This species occurs in most different open or woodland habitats of urban, agricultural or more natural landscapes (Seifert, 2018). *M. rubra* colonies are both polygynous and polydomous (Elmes, 1973). Single colony may often span multiple nest sites in rotten logs, plant pads, litter, soil or under stones (Groden *et al.*, 2005; Seifert, 2018). *M. rubra* is an omnivorous species, acquiring protein from a variety of prey (primarily invertebrate), and carbohydrates from homopterans and plant exudates (Cogni *et al.*, 2003; Seifert, 2018). The fruiting body of the truffle seems to be a suitable microhabitat for *M. rubra* to build a nest, especially since this species is the most hygrophilous Central European *Myrmica* species (Czechowski *et al.*, 2012). We can also assume that the fungus could also be a food source due to omnivorous nature of this ant. Our observation of a specimen of *S. debile* on the fruiting bodies can be explained by the accidental foraging of this species in the vicinity of truffles. They mainly foraging for dead or living invertebrates, occasionally feeding on dripped-off honeydew or elaiosomes (Seifert, 2018). It is a species of temperate deciduous forests in Europe, Crimea, and Caucasus (Czechowski *et al.*, 2012; Seifert, 2018). *S. debile* avoids water-logged and xerothermous habitats. The species forms not very numerous, generally monogynous colonies (Czechowski *et al.*, 2012) and prefers to nest under stones, in or under the litter layer, among roots, more rarely under dead wood (Czechowski *et al.*, 2012; Seifert, 2018).

The Forest Research Institute is the only scientific institution in Poland dealing with the study of all truffle-inhabiting insects groups in their entirety, and one of the few in the world (*e.g.* García-Montero *et al.*, 2004; Pérez-Andueza *et al.*, 2015). More often, scientists undertake projects devoted only to selected species of insects associated with truffles *e.g. L. cinnamomea*, *Ochodaeus berytensis* Petrovitz, 1965 or Bolboceratidae family (Fabra, 2003; Hochberg *et al.*, 2003; Byk *et al.*, 2016; Ćurčić *et al.*, 2019; Huchet *et al.*, 2022; Kilian *et al.*, 2022). Studies on this topic require close cooperation between mycologists and entomologists with narrow specializations (Rosa-Gruszecka *et al.*, 2023a). For some insect groups, such as the flies of the family Heleomyzidae, there are very few specialists who can identify specimens, and the studies described in the literature are mostly based on morphological analyzes of the insects (Woźnica, 2006; Woźnica, 2011; Woźnica and Kirk-Spriggs, 2021). The keys to recognizing beetles from the family Leiodidae are based (*e.g.* Daffner, 1982; Nunberg, 1987), among other things, on the structure of the male genitalia, and for this purpose the copulators should be dissected and prepared by clearing and maceration. Here, too, confusion occurs. For example, *L. cinnamomea* is often mentioned in the literature as the cause of fruiting body damage (Julià *et al.*, 2023; Marjanović *et al.*, 2023), while studies by the Forest Research Institute have shown that its twin species *L. oblonga* is just as common, but is never mentioned (Rosa-Gruszecka *et al.*, 2017). There are similar doubts about the 'truffle flies' from the *Cheilosia* genus, of which there are several very similar species (Ballester-Torres *et al.*, 2022). In order to dispel such kind of doubts, molecular analyzes should be required in the future (Rosa-Gruszecka *et al.*, 2023a).

Until now, adult insects collected in the field and reared in the laboratory were identified only by morphological methods. It should be emphasized that it requires laboratory facilities, special equipment (chambers), sacrifice of many truffles and constant monitoring for months or even years. The method described has proven to be effective in the area studied, but has significant drawbacks: it is very expensive, time-consuming and requires the assistance of decreasing number of entomological experts (Hochkirch *et al.*, 2022). The spread of genetic research would make possible to obtain more data on the biology of insects in a shorter time in quickly and inexpensively (Sharley *et al.*, 2004; Kirk *et al.*, 2013).

Conclusion

Knowledge of the insects that inhabit the fruiting bodies of truffles, their biology, ecology and mutual interactions is still very limited. This is partly due to the difficulties of observation (underground development of the fruiting bodies) and the need for collaboration between researchers with many narrow specializations in this type of study. Thanks to the breeding of insect larvae and collaboration with specialists, we have managed to obtain interesting results, but we have also realized that morphological methods alone are not sufficient for the future. The inclusion of molecular methods in research on this issue is necessary if we want to obtain complete information and accelerate work on this topic. It seems that continuing research in this area is the only way to protect truffle plantations in the future. Only a good knowledge of the biology and ecology of the insects that destroy the fruiting bodies, as well as knowledge of their role in the ecosystem, can allow the creation of a selective pest control system with the use of modern protection methods, such as periodically hung pheromone traps, means that disturb the swarm or attract the males away from the plantations.

Authors' contributions

A.R.-G. designed the research; A.R.-G., PS and D.H. performed sampling; A.R.-G., P.S., S.K., A.J.W., P.T. and I.S. did laboratory work; A.R.-G., S.K., A.J.W., P.T., I.S., D.H. and G.P. analysed data, discussed results and commented on the article. A.R.-G. led the writing and agrees to serve as the author responsible for contact and ensuring communication. All authors revised the manuscript.

Conflicts of interest

The authors declare no competing interests regarding the publication of this paper.

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STRESZCZENIE

Owady zasiedlające owocniki trufli letniej *Tuber aestivum* Vittad. w Polsce

Trufle *Tuber* spp. to grzyby wytwarzające podziemne owocniki, które rosną w różnego rodzaju formacjach roślinnych na całym świecie (zwykle w lasach i zaroślach) lub w specjalnych systemach rolno-leśnych (ogrodach/plantacjach trufli) kontrolowanych przez człowieka. Trufle są często zasiedlane przez liczne gatunki owadów, których larwy rozwijają się we wnętrzu owocników i uszkadzają je. Może to być przyczyną znaczących strat w komercyjnych uprawach tych jadalnych grzybów. Z drugiej strony bezkręgowce biorą udział w dyspersji zarodników trufli w przyrodzie. Niektóre owady można uznać za wskaźniki obecności trufli w środowisku leśnym. Mimo dziesięcioleci badań związki trufli i owadów są wciąż słabo poznane. Celem niniejszej pracy było poznanie składu gatunkowego owadów zasiedlających owocniki trufli letniej *Tuber aestivum* Vittad. Prace terenowe prowadzono w latach 2016-2017 na 4 naturalnych stanowiskach występowania trufli w południowej Polsce. Poszukiwania owocników prowadzono z pomocą wyszkolonego psa truflowego lub zbierano je losowo na powierzchniach badawczych. Dorosłe owady odławiano bezpośrednio z owocników za pomocą ekshaustora i umieszczano w eppendorfach z alkoholem. Zebrane owocniki trufli letniej przewieziono do laboratorium, zważono i sprawdzono pod kątem zasiedlenia przez owady (obecność otworów i śladów żerowania). Grzyby z cechami zainfekowania larwami owadów umieszczono w specjalnych pojemnikach w celu ich hodowli. Owady hodowano w komorze biologicznej o stałych parametrach (kontrolowano temperaturę i wilgotność) przez 2 lata i poddawano kontroli co 10-14 dni. Po przepoczwarczeniu osobniki zbierano do eppendorfów z alkoholem. Dorosłe owady zebrane w terenie i osobniki wyhodowane w laboratorium segregowano i oznaczano w oparciu o cechy morfologiczne. W trakcie badań zebrano i zbadano 584 owocniki trufli letniej. W 2016 r. owady zainfekowały 90 owocników trufli

(ok. 24,7%), natomiast w 2017 r. uszkodzone zostały 93 owocniki, co stanowiło 42,2% zbiorów. Łącznie zebrano 330 okazów owadów należących do 21 gatunków (tab. 1). Zidentyfikowano zgrupowania owadów zamieszkujących trufle, z 2 wyraźnie dominującymi gatunkami: muchą *Cheilosia soror* (Zetterstedt, 1843) z rodziny Syrphidae i chrząszczem *Colenis immunda* (Sturm, 1807) z rodziny Leiodidae (tab. 2). Wśród najczęściej występujących gatunków owadów zaobserwowano muchówki *Suillia affinis* (Meigen, 1830) i *S. pallida* (Fallén, 1820) oraz chrząszcze *Leiodes cinnamomea* (Panzer, 1793) i *L. oblonga* (Erichson, 1845). Po raz pierwszy opisano przypadek kolonizacji owocników trufli przez mrówki *Myrmica rubra* (Linnaeus, 1758). Wśród zidentyfikowanych owadów znalazły się 2 rzadkie gatunki chrząszczy z rodziny kusakowatych (Staphylinidae): *Atheta crassicornis* (Fabricius, 1792) i *A. gagatina* (Baudi di Selve, 1848) oraz muchówka z rodziny Heleomyzidae: *Suillia gigantea* (Meigen, 1830). Pionierskie badania prowadzone w Polsce przyczyniły się do znacznego poszerzenia wiedzy na temat owadów zasiedlających trufle, ich występowania i biologii, co jest niezbędne w ograniczaniu szkód powodowanych przez nie w produkcji trufli.