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RESEARCH

Mattesia cf. *geminata*, an ant-pathogenic neogregarine (Apicomplexa: Lipotrophidae) in two *Temnothorax* species (Hymenoptera: Formicidae)

Mustafa Yaman[1] · **Kadri Kıran**[2] · **Renate Radek**[3]

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Abstract
An ant-pathogenic neogregarine in *Temnothorax affinis* and *T. parvulus* (Hymenoptera: Formicidae) is described based on morphological and ultrastructural characteristics. The pathogen infects the hypodermis of the ants. The infection was mainly synchronous so that only gametocysts and oocysts could be observed simultaneously in the host body. Gametogamy resulted in the formation of two oocysts within a gametocyst. The lemon-shaped oocysts measured 11–13 µm in length and 8–10 µm in width. The surface of the oocysts is not smooth but contains many buds. A ring-shaped line containing rosary-arrayed buds line up in the equatorial plane of the oocyst. These specific characteristics were observed for the first time in neogregarine oocysts from ants. Polar plugs were recognizable clearly by light and electron microscopy. The oocyst wall was quite thick, measuring 775 to 1000 nm. Each oocyst contained eight sporozoites. The neogregarines in the two *Temnothorax* species show many similarities such as the size and shape of the oocysts, a relatively fragile gametocyst membrane, host affinity, and tissue preference. We identified these neogregarines as *Mattesia* cf. *geminata*, which is here recorded from natural ant populations in the Old World for the first time. All neogregarine pathogens infecting ants in nature so far have been recorded from the New World. We present the two ant species, *Temnothorax affinis* and *T. parvulus*, as new natural hosts for *M.* cf. *geminata*. Furthermore, the morphological and ultrastructural characteristics of the oocyst of *M.* cf. *geminata* are documented by scanning and transmission electron microscopy for the first time.

Keywords Ants · *Temnothorax affinis* · *Temnothorax parvulus* · Oocyst · Ultrastructure

Introduction

Entomopathogens contribute to the natural regulation of insect populations, as they naturally infect many insect species and transmit between insect populations (Lange and Lord 2012; Baki et al. 2021). Gregarines are common protistan entomopathogens in insects. Among gregarines, only the neogregarines are frequent causes of insect morbidity and mortality by generally invading the fat body and thus consuming their host's energy sources. Neogregarines have been isolated and identified from various insect orders including Coleoptera, Hymenoptera, Lepidoptera, and Siphonaptera (Buschinger and Kleespies 1999). Recently, studies on neogregarines have focused on the identification of new species (Yaman and Radek 2015, 2017), their presence in different insect groups, and their distribution in host populations (Yaman 2017).

Several neogregarines have been identified from different insect groups (Undeen and Vávra 1997), but studies on the neogregarine pathogens of ants (Formicidae) are still limited. Although there are current studies on faunistic or habitat types (Kiran and Karaman 2021; Stukalyuk et al. 2020, 2021), there is no further research on neogregarines from ants. Up to now, two members of the genus *Mattesia* and one undefined gregarine have been recorded from ants. The life cycle of *Mattesia* species in short is as follows

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✉ Mustafa Yaman
muyaman@hotmail.com

[1] Department of Biology, Faculty of Arts and Science, Bolu Abant İzzet Baysal University, Bolu, Turkey

[2] Department of Biology, Faculty of Sciences, Trakya University, Edirne, Turkey

[3] Institute of Biology/Zoology, Free University of Berlin, Working Group Evolutionary Biology, Königin-Luise-Str. 1-3, 14195 Berlin, Germany

(Perkins et al. 2000): oocysts are taken up orally; hatched sporozoites migrate to the fat body and develop into meronts; a first generation of micronuclear merozoites spreads the infection within the fat body; then macronuclear merozoites are formed and differentiate into gamonts; pairs of gamonts form gametocytes; after nuclear divisions including meiosis two zygotes emerge; each zygote transforms to a thick-walled oocysts with polar plugs and 4–8 internal sporozoites. *Mattesia geminata* from *Solenopsis geminata* is the only species from an ant identified at the species level (Jouvenaz and Anthony 1979). Later, it was also recorded from two *Leptothorax* species from the *L. muscorum* complex (Buschinger et al. 1995; Kleespies et al. 1997). This pathogen has also been experimentally transmitted to some other ant species from the genus *Leptothorax* (Buschinger and Kleespies 1999; Kleespies et al. 1997). One yet undescribed *Mattesia* species, which is morphologically clearly different from *M. geminata*, was recorded from *Solenopsis invicta* (Pereira et al. 2002). In addition to the two *Mattesia* species, one undefined gregarine pathogen was recorded from the Australian bull ant, *Myrmecia pilosula* (Crosland 1998). In the present study, we report *M. geminata* in populations of *Temnothorax affinis* and *T. parvulus* for the first time. Furthermore, we are the first to describe the morphological and ultrastructural characteristics of the oocyst of *M. geminata* by scanning and transmission electron microscopy.

Material and methods

Insect samples

The study was carried out on ant samples stored in 70% ethanol. Pupae and adults of *Temnothorax affinis* and *T. parvulus*, collected in 2012 and 2013 in Artvin and Gümüşhane, Turkey, respectively were provided by one (K.K.) of authors from his collections, originally collected from natural populations.

Pupae and adults of *Temnothorax* spp. were collected by hand from mixed forests in the northeast Black Sea region of Turkey. The insects were put into glass bottles to prevent possible contamination. They were brought to the laboratory and dissected as soon as possible. Detailed information about the collecting sites is given below:

Host species: *Temnothorax affinis* (Mayr 1855)

Site: Artvin-Şavşat-Kirazlı Village, 1662 m, N 41°15′06″, E 42°29′29″ and Kelkit-Babakonağı Village, 1800 m, N 40°03′24″, E 39°21′57″

Collection date: 12 August 2012 and 21 August 2013

Habitat: Mixed forest with spruce, elm, and maple (*Acer pseudoplatanus*), no slope, grasslands and spruce forests around

Microhabitat: Rotten beech branch

Examined samples: Twenty adults (worker) and three pupae

Host species: *Temnothorax parvulus* (Schenck 1852)

Site: Gümüşhane-Kelkit-Sadak Village, 1702 m, N 40°01′47″, E 39°38′05″

Collection date: 20 August 2013

Habitat: Oak forest (with very few *Pinus sylvestris*), 20° slope

Microhabitat: In soil

Examined samples: Twenty adults (worker) and two pupae

Light microscopy

Ant samples were examined for symptoms of infection under a stereo microscope. Then, each ant was dissected in insect Ringer solution and wet smears were examined for the presence of a neogregarine infection under a light microscope at a magnification of 200–1000 × (Yaman 2017). When an infection was found, the slides were air-dried and fixed in methanol for 2–5 min. The slides were then washed with distilled water and stained for approximately 10 h in freshly prepared 5% solution of Giemsa stain (stock solution, Carloerba, No. 6B712176C). Afterwards, the slides were washed in running tap water, air-dried, and re-examined under the microscope (Undeen and Vávra 1997). Detected gametocysts and oocysts were measured and photographed using Optika B-293PLi microscope with a digital camera and Optika Proview Digital Camera Software. The data of fresh oocysts are presented as mean values ± standard deviation. Parts of the infected specimens were used for preparing samples for scanning (SEM) and transmission (TEM) electron microscopy.

Electron microscopy

Samples for transmission electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h, rinsed in cacodylate buffer, post-fixed in 1% OsO_4 for 2 h, and rinsed in cacodylate buffer. After dehydration in an increasing ethanol series, parts of infected beetles were embedded in Spurr's resin (Spurr 1969). Ultra-thin sections were mounted on Pioloform-coated copper grids which were stained with saturated uranyl acetate and Reynolds lead citrate (Reynolds 1963). They were then examined with a Philips EM 208 transmission electron microscope. For scanning electron microscopy, tissue samples stored in distilled water were crushed in a drop of water, slightly spread on a cover glass, and air dried. After sputtering with gold, the samples were observed in a FEI Quanta 200 scanning electron microscope.

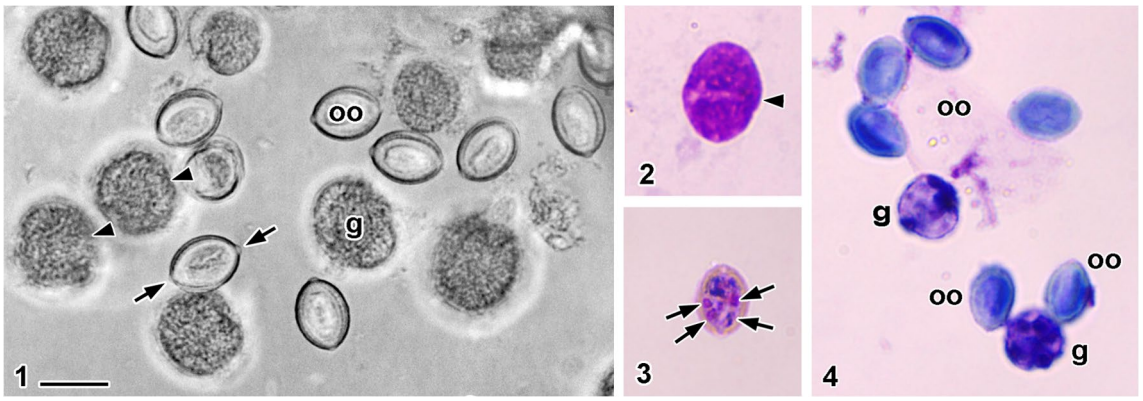

Fig. 1–4 Oocysts and developing gametocysts of *Mattesia* cf. *geminata* in the light microscope. **1** Fresh preparation, **2–4** Giemsa staining. **1** The arrows point to polar plugs of oocysts (oo). Arrowheads show the cytoplasmic division within the developing gametocysts (g). **2** Bilobed developing gametocysts. Arrowhead shows the division plane. **3** Oocyst with sporozoite nuclei (arrows). **4** Oocysts next to developing gametocysts. Bar for **1–4** = 10 µm

Results

There were no obvious external signs of infection under the stereo microscope. Under the light microscope, neogregarine infections were found only in four pupae of the two *Temnothorax* species, *T. affinis* and *T. parvulus*, collected from two natural populations. The typical lemon-shaped oocysts and early gametocysts of the pathogen were observed in the hypodermis (Figs. 1, 2, 3, and 4). The stages were also found dispersed in the hemolymph (Fig. 1) but did not infect tissues other than hypodermis. The infection was synchronous so that only early gametocysts and oocysts could be observed at the same time, but not other, vegetative stages (Figs. 1, 4). This was true for infected pupae. We could not find any infection in twenty adult (worker) individuals for each ant species from two colonies.

The gametocyst wall appeared to be fragile and not resistant so that mature oocysts were rarely observed in pairs. The oocysts were lemon-shaped and had a quite uniform size. Fresh oocysts ($n = 50$) measured 12.26 ± 0.62 (11.04–13.29) in length and 9.04 ± 0.37 (8.20–9.87) µm in width in *T. affinis* and Giemsa-stained oocysts measured 11.29 ± 0.53 (10.5–12.36) in length and 7.53 ± 0.63 (6.35–8.57) µm in width in *T. affinis* and 11.27 ± 0.78 (10.21–12.3) in length and 7.49 ± 0.47 (6.5–8.32) µm in width in *T. parvulus.* Polar plugs were recognizable clearly by light microscopy (Fig. 1). Each oocyst contained eight sporozoites (Fig. 3). Detailed examination of the oocyst by scanning electron microscopy showed that clearly visible annular ridges surround the polar plugs (Figs. 5, 6, and 7).

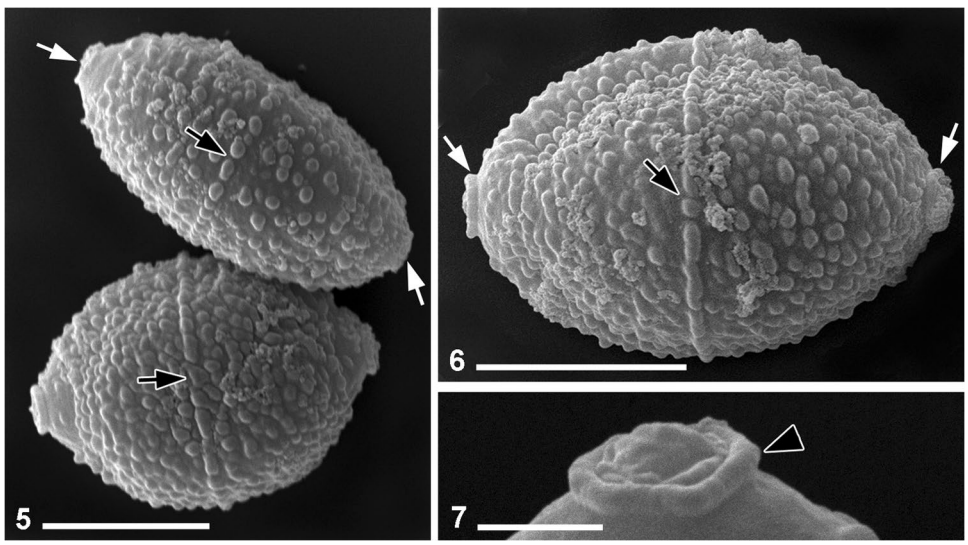

Fig. 5–7 SEM micrographs of oocysts of *Mattesia geminata*. **5**, **6** Whole oocysts. Note the buds on the surface and a ring-shaped line containing rosary-arrayed buds (black arrows) in the equatorial plane of the oocyst. White arrows point to polar plugs. **7** Polar plug of the oocyst surrounded by a circular ring (arrowhead). Bars = 5 µm for **5** and **6**, 1 µm for **7**

The surface of the oocysts is not smooth but contains many buds (Figs. 5, 6, and 7). Additionally, a ring-shaped line containing rosary-arrayed buds line up in the equatorial plane of the oocyst (Figs. 5, 6). The structure of the oocyst was well seen in the transmission electron microscope (Figs. 8, 9, 10, 11, and 12).

The polar plug measured 1000 to 1100 nm in diameter, excluding the annular ridge. The oocyst wall with the surface buds was quite thick, measuring 775 to 1000 nm (Figs. 8, 9, 11, 12). The wall consisted of two layers, a very thin, more electron-dense outer layer and a thick, less electron-dense inner layer. The ring surrounding the polar plug was clearly visible in a longitudinal section of the oocyst (Fig. 10).

Discussion

Taxonomic identity

Light and electron microscopic observations revealed that the neogregarine pathogen detected in *Temnothorax* species has typical characteristics of the members of the genus *Mattesia* Naville, 1930 (family Lipotrophidae) as two oocysts within a gametocyst and eight sporozoites within each oocyst (Jouvenaz and Anthony 1979; Kleespies et al. 1997; Perkins et al. 2000; Undeen and Vávra 1997). *Mattesia* exclusively infects insects. To date, two *Mattesia* species, *M. geminata* and *Mattesia* sp., and one undefined gregarine infection have been observed in ants (Table 1) (Jouvenaz and Anthony 1979; Buschinger et al. 1995; Pereira et al. 2002). All infected ant hosts belong to the subfamily Myrmicinae

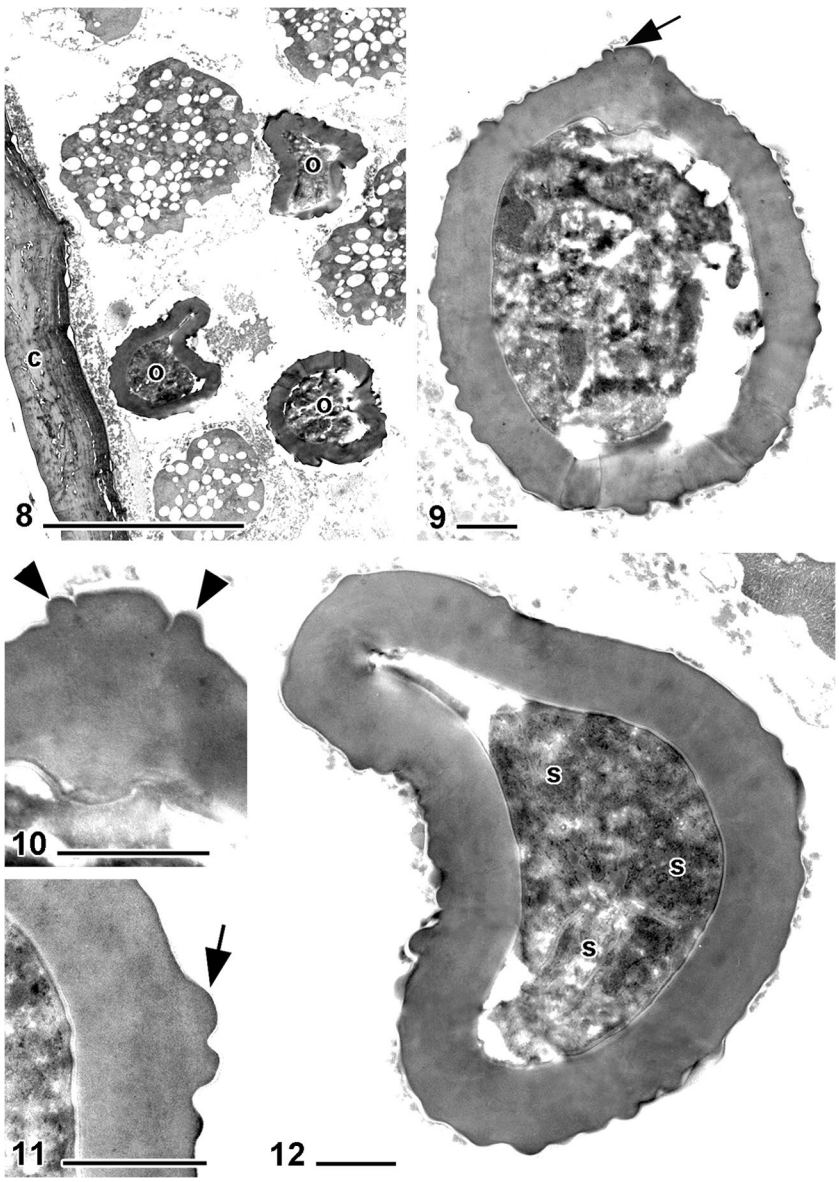

Fig. 8–12 TEM micrographs of oocysts (o) of *Mattesia* cf. *geminata*. **8** Infection in the hypodermis under the cuticle (c). **9** Oblique section of whole oocyst. **10** Longitudinally sectioned polar plug of oocyst in **9**, bordered by a circular ring (arrowheads). **11** Oocyst wall composed of a thin, electron-dense outer layer and thick medium-dense inner layer. Wall is not smooth but contains buds (arrow). **12** Cross section of a whole oocyst, containing sporozoites (s). Bars = 10 µm for **8** and **9**, 1 µm for **10**–**12**

Table 1 Neogregarine species described from ants (Formicidae: Myrmicinae)

Pathogen species	Oocyst size, length × width, mean (µm)	Infected organ	Host life stage	Host	Location	References
Undefined gregarine	13 (in length)[F]	–	Adult	*Myrmecia pilosula*	Australia	Crosland 1998
Mattesia sp.	18.7 ± 0.80 × 10.8 ± 0.80[F]	Hypodermis	Adult	*Solenopsis invicta*	FL, USA	Pereira et al. 2002; Valles and Pereira 2003
Mattesia geminata	11.3 (10.5–12.5) × 7.9 (7–8.5)[F]	Hypodermis	Pupa	*Solenopsis geminata*	Southeastern USA	Jouvenaz and Anthony 1979
Mattesia geminata	12.5–13 × 8.5–9[F]	Hypodermis	Pupa	Two *Leptothorax* species from *L. muscorum* complex	MT, USA	Buschinger et al. 1995
Mattesia geminata	12.26 ± 0.62 (11.04–13.29) × 9.04 ± 0.37 (8.20–9.87)[F] 11.29 ± 0.53 (10.5–12.36) × 7.53 ± 0.63 (6.35–8.57)[G]	Hypodermis	Pupa	*Temnothorax affinis*	Artvin, Turkey	In this study
Mattesia geminata	11.27 ± 0.78 (10.21–12.3) × 7.49 ± 0.47 (6.5–8.32)[G]	Hypodermis	Pupa	*Temnothorax parvulus*	Gümüşhane, Turkey	In this study

[F]Fresh, [G]Giemsa-stained

(Formicidae: Hymenoptera). The typical infection site in all recorded *Mattesia* infections is the hypodermis (Fig. 8; Table 1). Large gametocysts with numerous oocysts (> 70) are typical for the undefined gregarine of the Australian bull ant *Myrmecia pilosula*, and thus this pathogen is clearly distinct from the genus *Mattesia* (Crosland 1998). Oocysts of the undescribed *Mattesia* species from *Solenopsis invicta* are larger than the ones of *M. geminata* and may thus belong to new species (Pereira et al. 2002). The oocyst size of the neogregarine found in our study closely resembles the oocyst size of *M. geminata*.

The host affinity is generally recognized as a valid taxonomic character for differentiating neogregarine species from insects (Jouvenaz and Anthony 1979; Kleespies et al. 1997; Levine 1988; Lord 2003). However, the host specificity of *M. geminata* in infection experiments using different ant species as hosts was ambiguous. While the authors were not able to transfer this pathogen from *Solenopsis geminata* to *S. invicta*, they succeeded in infecting some species belonging to other genera of the subfamily Myrmicinae, such as *Leptothorax acervorum*, *L. muscorum*, *L. unifasciatus*, *L. recedens*, and *Harpagoxenus sublaevis* (Buschinger and Kleespies 1999).

The neogregarine pathogen from *Temnothorax* species shows many matches with *M. geminata* (Jouvenaz and Anthony 1979) such as size and shape of oocysts, relatively fragile gametocyst membrane, host affinity, and tissue preference. The dimensions of the oocysts are a good criterion for comparing neogregarines from different insect hosts (Pereira et al. 2002). As seen in Table 1, the neogregarine in this study shows a considerable difference in oocyst size compared to *Mattesia* sp. from *S. invicta*. The oocyst of the pathogen presented here is much smaller, especially in length (12.26 ± 0.62 (11.04–13.29 µm)), than the oocyst of *Mattesia* sp. (18.7 × 10.8) from *S. invicta* (Pereira et al. 2002). However, it is relatively similar to the oocyst of *M. geminata* from *Solenopsis geminata* (11.3 × 7.9; Jouvenaz and Anthony 1979) and that from two *Leptothorax* species from the *L. muscorum* complex (13.8 × 9.3 µm; Buschinger et al. 1995). Except for insignificant variations in size between the described *M. geminata* oocysts, there are no morphological differences justifying the creation of a new species for the neogregarine from the *Temnothorax* species. Thus, we conclude that the reported pathogen in natural populations of two *Temnothorax* species is identical to *M. geminata* and thus, *T. affinis* and *T. parvulus* should be added to the list of hosts for this pathogen. However, we named the identified neogregarine pathogen as *Mattesia* cf. *geminata*. Confer (cf.) is used for the pathogen species as it cannot be supported with a comparison of 18S rRNA sequences of neogregarine pathogens from ants to confirm the pathogen clearly as *M. geminata* recorded from original host.

Ultrastructure

All descriptions of *M.* cf. *geminata* were based on light microscopic observations to date, but in the present study, we provided ultrastructural information in addition to detailed morphological analysis. In two recent studies, we presented fine structural features of oocysts from two species of different neogregarine genera, *Mattesia weiseri* from *Dendroctonus micans* (Coleoptera: Curculionidae) (Yaman and Radek 2015) and *Ophryocystis anatoliensis* from *Chrysomela populi* (Coleoptera: Chrysomelidae) (Yaman and Radek 2017). Both species have oocysts with a smooth surface and without annular ridges around the polar plugs. In contrast, unlike *M. geminata*, clearly visible annular ridges that surround the polar plugs further distinguish *M. weiseri* and *O. anatolensis* from *M.* cf. *geminata*.

Life cycle and pathogenicity of *Mattesia* species

In the original study, Jouvenaz and Anthony (1979) found *M. geminata* in the pupae of *S. geminata*, and not in adults from infected colonies. They showed that the reason for the absence of pathogens in adults was that the pupae died and could not mature as the disease appeared to be fatal. Later Buschinger et al. (1995) also found *M. geminata* in the pupae of *Leptothorax* ants. We also found *M.* cf. *geminata* only in the pupae of *Temnothorax* species and not in the adults from the infected colonies. Other neogregarine species do not seem to cause mortality of ant pupae. Crosland (1998) reported an undefined gregarine pathogen in adults of the Australian bull ant *Myrmecia pilosula*, and Pereira et al. (2002) found *Mattesia* sp. in adults of the red imported fire ant *S. invicta* in Florida. Buschinger and Kleespies (1999) proved experimentally that *M. geminata* can infect several other ant species from the subfamily Myrmicinae. According to these studies, there is a possibility that *M. geminata* can infect adult ants under natural conditions.

Neogregarines infect insects, and many species are pathogenic to their hosts. They are usually found in the fat body, hemolymph, hypodermis, gut, or Malpighian tubules. The most common site of infection is the fat body. However, neogregarines found in ants generally show a tissue specificity for the hypodermis. *M. geminata* seems to even have different tissue affinities in different ant hosts (Kleespies et al. 1997). In *Leptothorax* species, e.g., it first multiplies in the hemolymph before it enters the hypodermis and fat body (Kleespies et al. 1997), while in *S. geminata* it only infects the hypodermis and oenocytes (Jouvenaz and Anthony 1979). In *Temnothorax*. we found the neogregarine in fat body, hypodermis, and hemocoel at the same time. Neogregarine infections in ants may cause specific symptoms. The earliest pathogenic symptom of a *M. geminata* infection in *S. geminata* appears as an irregularity of the developing eyes of the pupa, followed by melanization of the cuticle, and then the entire pupa becomes solid black (Jouvenaz and Anthony 1979). *M. geminata* also causes an irregular eye pigmentation of the gray pupae of *Leptothorax* species (Buschinger et al. 1995). An undescribed *Mattesia* species in the red fire ant, *S. invicta*, causes a typical yellow-orange colored head and sometimes thorax and was therefore designated *Mattesia* "yellow-head disease" (YDS) (Pereira et al. 2002). According to Crosland (1998), a strong infection with the undefined gregarine in the Australian bull ant, *Myrmecia pilosula*, interferes with the normal darkening of cuticle in the pupal stage, leading to abnormal brown ants. In contrast to these symptoms in different ant species, we did not observe any symptoms in the infected pupae of the two investigated *Temnothorax* species.

The ant genus *Temnothorax* Mayr is one of the most species-rich genera in the subfamily Myrmicinae (Rasheed et al. 2020), and *Leptothorax* and *Temnothorax* are closely relative genera. *Leptothorax* has a distribution in North America, Western Asia, and the northern parts of Europe, while *Temnothorax* has a general holarctic distribution. Species of both genera live in very diverse habitats—from cold forests in the forest-tundra zone to the dry steppe or even semi-desert and can be found in the same habitat (Bolton 2003; Radchenko 2004). One of the interesting results of our study is that the locations (Artvin and Gümüşhane, Türkiye) of the *Temnothorax* species infected with *M.* cf. *geminata* are from the Old World. All neogregarine pathogens infecting ants in nature so far have been recorded from the New World (Table 1).

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Author contribution Mustafa Yaman conducted experiments, identified the neogregarine pathogen, and wrote the manuscript. Kadri Kıran provided ant samples. Renate Radek conducted experiments and improved the manuscript.

Data availability All of the data generated and analyzed during this study are included in this published manuscript.

Declarations

Ethical approval This is not applicable to the present manuscript.

Consent to participate This is not applicable to the present manuscript.

Consent for publication All authors reviewed and approved the final version of the manuscript.

Conflict of interest The authors declare no competing interests.

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