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The Biology of *Szelenyiopria talitae* (Hymenoptera: Diapriidae): Larval Parasitoid of the Leaf-Cutting Ant *Acromyrmex subterraneus* (Hymenoptera: Formicidae)

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Abstract

The biology of a koinobiont parasitoid of leaf-cutting ant larvae, *Szelenyiopria talitae* (Hymenoptera: Diapriidae), was studied from naturally infested *Acromyrmex subterraneus* (Hymenoptera: Formicidae) nests. Nests were collected in the field from the Atlantic rainforest biome in the state of Rio de Janeiro. A total of fifty-three nests were collected from 2015 to 2018. Parasitized nests were only found during the months of September and October. Approximately 22% of the nests collected over a four-year period were found to have been parasitized by *S. talitae*. The mean within-nest parasitism rate was 66.3%. This diapriid displayed both solitary (14%) and gregarious parasitism (86%), with up to a maximum of 12 parasitoids developing within a single host. Gregarious parasitism with two (29%) or three (21%) *S. talitae* per host was most frequently observed. There was a positive correlation between the number of parasitoids per host and host size (dry weight), indicating that *S. talitae* females oviposited a higher number of eggs in larger hosts. There was also a negative correlation between *S. talitae* pharate adult size and the number of parasitoids per host, which could have been caused by sibling competition for limited host resources. The high levels of parasitism seen here had a debilitating effect on the colonies. *Acromyrmex subterraneus* is a serious pest in Brazil, and these studies lay the foundation for understanding the impact of *S. talitae* on ant populations.

1. Introduction

Leaf-cutting ants (Attini: Formicidae) are important pests in many agro-systems throughout the Neotropics [1]. Leaf-cutting ants cause severe damage due to the large quantities of fresh leaves they cut and carry to the nest, which are used to cultivate their symbiotic fungus *Leucoagaricus gongylophorus* (Möller) Singer (Agaricales: Basidiomycota). Chemical baits continue to be the most commonly used control method for leaf-cutting ant management [2]. However, such methods cause negative impacts on human health and the environment [3]. In addition, the use of chemical insecticides may promote insect resistance and affect nontarget organisms [4]. Alternative approaches are therefore urgently needed, such as the use of biological control. However, currently there are no commercially available biological control agents for the management of these pests. Studies of the natural enemies of leaf-cutting ants could lead to the development of viable biological control strategies.

An example of the use of parasitoids against ant pests is the phorid flies (*Pseudacteon* sp. Diptera: Phoridae), which are currently deployed in the management of fire ants. Phorids parasitize fire ant adults, resulting in decapitation of the ants [5] [6]. The information available on this host-parasitoid interaction is an important reference as to what should be studied in the development of a biological control program for leaf-cutting ants when considering the use of parasitoids.

Phorid flies also parasitize adult leaf-cutting ants. Although phorids parasitize only low percentages of leaf-cutting ants, they are still considered as important natural biological control agents. Parasitism rates of worker ants are normally between 1% - 5%, however, in the case of *Eibesfeldtphora curvinervis*, parasitism rates of up to 15% of foraging *Atta cephalotes* have been observed [7]. Phorids attack adult leaf-cutting ants while they are foraging on trails [8] and, therefore, these parasitoids do not need to enter the nests. Under certain circumstances, the presence of these flies can significantly reduce the foraging behavior of the ants [9] [10]. Many species of phorids oviposit eggs in the heads or abdomens of worker ants, with the parasitoid larvae slowly developing inside the host, consuming the internal tissues, resulting in death [7] [11]. Phorids normally lay one egg per host, but *Apocephalus attophilus* can oviposit up to 14 eggs per host ant [12]. The development of multiple *A. attophilus* larvae inside the hosts' head was not due to super-parasitism because all larvae were observed to be at similar stages of development and the flies emerged from the host on the same day.

As well as phorid flies, diapriid wasps (Hymenoptera, Proctotrupoidea, Diapriidae) are also natural enemies of Attini. Although most known species of Di-

apriidae evolved as primary parasitoids of dipteran pupae [13] [14], diapriids are also parasitoids of ant larvae [15]. Diapriid parasitoids of ant larvae are koinobiont endo-parasitoids with a solitary or gregarious habit [15] [16]. These symphyles exhibit morphological and behavioral adaptations for co-existence with the ants, which helps them avoid detection and/or aggression by their hosts [15]. The first record of a diapriid attacking ant brood was in 1982, when *Plagiopria passerai* was observed parasitizing queen cocoons of *Plagiolepis pygmaea* (Hymenoptera: Formicinae) [17].

Although New World diapriine wasps are abundant and diverse, their biology has been rarely studied, especially in the case of species that attack higher Attini. In one of the few studies of diapriid parasitoids attacking fungus-cultivating ants, *Acanthopria* and *Mimopriella*, were found parasitizing *Cyphomyrmex* larvae [18]. Six diapriine wasp morphotypes, two of them of the genus *Mimopriella*, one *Oxypria*, two *Szelenyiopria* and one *Acanthopria* were found parasitizing larvae of the fungus-growing ant *Trachymyrmex* cf. *zeteki* [19]. Diapriids that attack higher Attini display both solitary and gregarious habits [20] [21].

The diapriid studied here, *Szelenyiopria talitae*, was first identified by Loiácono *et al.* [22] from material supplied by our research group. Their study contributed to the taxonomy and classification of this new species of diapriid. The description of the species included biological data based on a single nest, with observations of the coloration of the parasitized hosts and the occurrence of solitary and gregarious habits, stating that the majority of the ant larvae were parasitized by solitary wasps.

In the current study, we aimed to understand the dynamics of *S. talitae* parasitism of natural populations of *A. subterraneus*, investigating parasitism rates and observing the development of this parasitoid. This study lays the ground work for evaluating the potential of *S. talitae* as a biological control agent of *A. subterraneus* and this interaction is also an interesting model for the study of parasitoid biology.

2. Material and Methods

2.1. Field Site Characteristics

The data presented here is from field collected material obtained during the months of September and October from 2015 until 2018. The collection site was located in the municipality of Bom Jardim, Rio de Janeiro State, Brazil (22˚09'07.0"S and 42˚25'08.0"W). This site is 650 m above sea level and has a CWA climate (Köppen-Geiger), with a subtropical dry winter, an average temperature of 20.1˚C and rainfall of 1516 mm per year. The area is located on farmland surrounded by Atlantic Rainforest. Nests were collected from an area of approximately 2 hectares.

2.2. Nest Collection, Maintenance and Observation

Individual ant nests were accessed by soil excavation to collect the maximum

amount of material possible, including the fungus garden, larvae, workers, queens, and associated organisms. Each nest was placed in a plastic container with a perforated lid partially covered with cotton wool soaked in water to maintain humidity. These containers were transported to the Myrmecology Unit of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF) in Campos dos Goytacazes, Rio de Janeiro, Brazil.

Nests were then placed in rooms maintained at 25˚C ± 2˚C, 75% ± 5% relative humidity, and a photoperiod of 12:12 h L:D. Maintenance techniques of the nests providing a foraging arena, containers for fungus chambers, and waste disposal areas, were adapted from Della Lucia *et al.* [23]. Fresh *Acalypha wilkesiana* and *Ligustrum lucidum* leaves were placed in the foraging arena daily. All nests collected and used in the experiments here contained queens.

2.3. Obtaining Parasitized ant Larvae and Evaluating Parasitism Parameters

Inspections of the fungus gardens were made daily following field collection with the aid of a stainless-steel spatula and forceps. The presence of late stage diapriid parasitized larvae in the brood chambers was easy to observe with the naked eye as these larvae were darker than apparently healthy ones (**Figure 1(C)**).
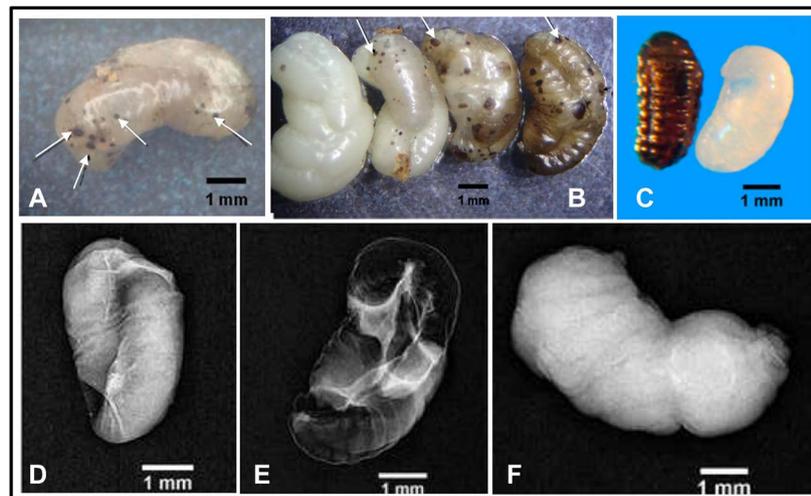

Figure 1. *Szelenyiopria talitae* pre-pupal and pupal phases of development within *Acromyrmex* larvae. (A) Early melanization of *S. talitae* pre-pupae, observed through the translucent host integument. White arrows mark the early melanization of the parasitoid pupal cuticle; (B) Four parasitized host larvae showing increasing levels of melanization from left to right; 1st host with six gregarious parasitoids before melanization had initiated; 2nd with 4 parasitoids at the start of the melanization process; 3rd and 4th with four parasitoids per host at a more advanced stage of parasitoid development; (C) A completely melanized solitary parasitoid pupa and a non-parasitized larva; (D) X-ray microscopy showing two parasitoids at the pre-pupal phase prior to complete melanization; (E) Two parasitoids at a slightly more advanced stage of pre-pupal development coinciding with increased melanization of the parasitoid integument; (F) Example of X-ray image of a control (non-parasitized) larva. Scale bar: 1 mm.

The average parasitism rate was obtained from a total of 53 nests collected between the years 2015 and 2018. Parasitism rates were calculated from the total number of nests collected during each yearly field trip (4 trips per year) and the number of nests subsequently found to be infested with *S. talitae*, evaluated 2 to 6 weeks after establishment in the Myrmecology Unit.

Six nests were then used for analyses of within-nest parasitism rates. Larvae were visually quantified as apparently healthy or parasitized and this information was used to calculate the within-nest parasitism frequency. Separation of parasitized and healthy larvae was carried out with the aid of a hand-held magnifying glass. For this process, it was necessary to fragment the fungus garden and these nests did not recover from this destructive process. To confirm clutch size (number of parasitoids per host), all melanized larvae were dissected.

The dry weight of individual parasitized ant larvae from one colony collected in 2017 was evaluated following drying in an incubator at 70°C for 72 hours. The dry weight was then determined using a Sartorius M5/SE2 microbalance. Dry weights of healthy larvae of a range of different sizes were also determined (Supplementary data Figure S1 and Table S1). The sample number (n) for each solitary or gregarious cohort (2 to 8 parasitoids per host = clutch size) varied between 14 and 32 for host dry weight determinations and for pharate adult size measurements (see Supplementary data Table S2).

Pharate adult parasitoids (n = 11 to 77 per clutch size group; N = 329, see Supplementary data Table S2) from both solitary and gregarious hosts (2 - 8 parasitoids per host) were removed from the host by dissection and length measured using a calibrated microscope eye-piece. X-ray microscopy was carried out using a Faxitron LX-60 X-ray microscope (Faxitron X-Ray Corp., Wheeling, IL, USA) and images were captured with a digital camera. This approach enabled the observation of parasitoid development in a non-destructive manner.

Specimen samples of *S. talitae* are currently deposited in the UENF Entomological Museum, the State University of Paraná Entomology Museum (Brazil), the Museum of Zoology of the University of São Paulo (Brazil) and the Entomology Division of La Plata Museum in Argentina. All diapriid parasitoids collected during the current study were verified as *S. talitae* by Denise Moreira.

2.4. Statistical Analysis

The statistical analysis of the frequency of solitary and gregarious parasitism (number of parasitoids per host = clutch size) was performed by calculating the average and the standard deviation of results obtained from individual nests. The results were tested for normality using Lilliefors test (P ≤ 0.05) and the homogeneity of the deviations was tested using the Cochran & Bartley test (P ≤ 0.05). The means were compared using a one-way ANOVA followed by the Least Significant Difference test (T ≤ 0.05; n = 24; LSD = 5.81).

The relationship between clutch size and parasitoid developmental parameters was evaluated by linear regression. Number of parasitoids per host (clutch size),

an independent variable, was associated with parasitized larval dry weight (mg) or pharate adult length (mm). The levels of significance of the models were determined by ANOVA.

3. Results

3.1. Parasitism Rates

From a total of 53 nests collected, twelve (mean 22.6%; range 14.3% to 29.4%) were found to have been parasitized (Table 1). The within-nest parasitism rates were evaluated from six nests (Table 2). The total mean number of larvae in these nests was 1145 (ranging from 639 to 1822) and the majority of the larvae (66.3% ± 12.9%) had been parasitized, ranging from 54.6% to 82.8%.

Figure 1 demonstrates the different developmental stages of the parasitoids within the host. The time from the first observations of parasitized larvae in the nests, identified by a gradual melanization of the parasitoid pupae visualized through the translucent host integument to parasitoid adult emergence, was estimated to take 22.8 (±9.9) days (data obtained from nests shown in Table 2). In the more advanced parasitoid pharate adult stages (Figures 2(A)-(C)) there is a gradual absorption/degradation of an electron opaque "capsule" which initially surrounds the parasitoid. It is interesting to note the orientation of the parasitoids in hosts parasitized by two *S. talitae* larvae, when parasitoids were always aligned in opposite directions (Figure 2(C)), indicating the optimal use of physical space within the host. Figure 2(D) shows a fully developed solitary adult parasitoid, ready to emerge and Figure 2(E) shows the parasitoid's head emerging from the host. Figure 2(F) shows a host parasitized by ten *S. talitae* in the

Table 1. Parasitism rates of the diapriid *Szelenyiopria talitae* in nests of *Acromyrmex subterraneus* collected over a 4 year period.

Collection date	Total nests	Parasitized Nests	
		n	%
10/2015	14	2	14.3
09/2016	13	3	23.1
10/2017	9	2	22.2
09/2018	17	5	29.4
Mean parasitism frequency (%) ± SD			22.6 ± 6.16

Table 2. Details of *Szelenyiopria talitae* parasitism of six *Acromyrmex subterraneus* nests.

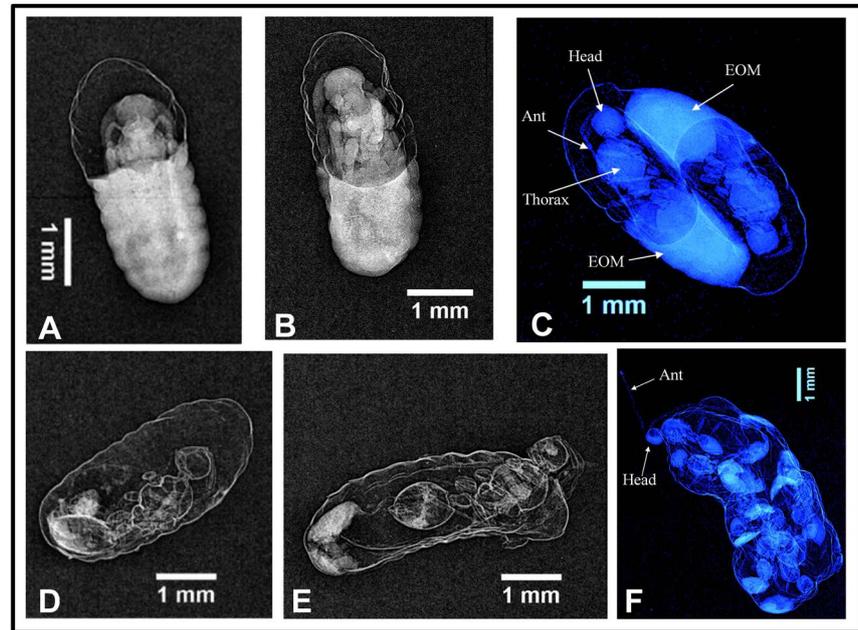| Nest	Fungus garden volume (L)	Total number of larvae	Healthy larvae	Parasitized larvae	Parasitism rate (%)
1	3	1822	827	995	55
2	5	1002	441	561	56
3	10	639	110	529	83
4	14	1329	616	713	54
5	14	1063	292	771	72
6	14	1014	223	791	78
Mean ± SD	10 ± 4.9	1144.8 ± 398.1	418.2 ± 266.8	726.7 ± 170.2	66.3 ± 12.9

Figure 2. X-ray microscopy images of *Szelenyiopria talitae* pharate-adult development and emergence. (A) Solitary parasitoid during early pharate adult development with adult structures visible. An opaque material covers a large part of the developing parasitoid; (B) More advanced pharate adult showing a reduction in the opaque material covering the abdomen (EOM); (C) Gregarious parasitism with two *S. talitae*; (D) Fully developed pharate-adult; (E) Emergence of a solitary adult with the head capsule outside the host; (F) Gregarious parasitized host with the emergence of one of the adult parasitoids, with the head breaking through the host cuticle. Scale bar: 1 mm. EOM: electron opaque material; Ant: Antenna.

final stages of development, with one of the adult parasitoids with its head breaking through the host cuticle.

3.2. Solitary and Gregarious Parasitism Rates

The frequency of solitary and gregarious parasitism was evaluated from 4 nests collected between 2015 and 2018. The results are shown in **Figure 3**. Solitary parasitism was observed in 14.2% (±7.3 SD) of the larvae which had been attacked by *S. talitae*. The highest frequencies of gregarious parasitism were observed when two (29% ± 9.8%) or three (21.5% ± 6.1%) parasitoids attacked individual host larvae. Gregarious parasitism was observed with up to a maximum of 12 parasitoids per host, but this was only seen at very low frequencies. The data for 9 to 12 parasitoids per host were not included in **Figure 3** as the combined frequency was only approximately 5%. Statistical analysis of the data showed that the frequency of gregarious parasitism with two parasitoids per host larvae was significantly higher than all other gregarious or solitary frequencies.

3.3. Influence of the Number of Parasitoids Per Host on Development

There was a positive and significant correlation between the host dry weight
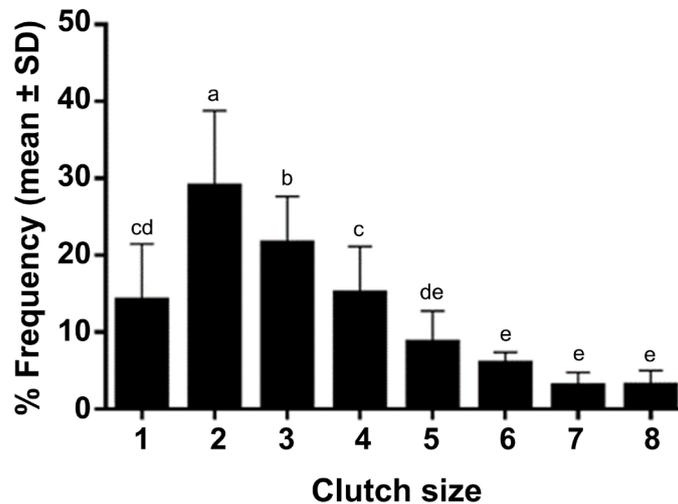
Figure 3. Frequency of solitary and gregarious parasitism evaluated in four *Acromyrmex subterraneus* nests. Note: The results were shown as the mean frequency (±SD) of each clutch size (number of parasitoids per host larvae). Different letters above the columns indicated statistical differences using ANOVA followed by the Least Significant Difference test at the 5% level.

(mg) and the number of parasitoids per host (clutch size) (F = 259.5; p < 0.0001) (**Figure 4(A)**). There was also a significant correlation (F = 34.727; p < 0.0015) between the length (size mm) of the adult parasitoids and the number of parasitoids per host (**Figure 4(B)**).

4. Discussion

An understanding of host-parasitoid interactions is not only of interest from a biological standpoint but is also important when considering the possibility of using parasitoids to control pest species. The results shown here indicated that *S. talitae* is a candidate for the biological control of *A. subterraneus* due to the high levels of parasitism, resulting in colony collapse.

Yearly collections of *A. subterraneus* nests were carried out between 2015 and 2018. The results showed that on average 22% of these nests had been parasitized by *S. talitae*. These results were similar to those seen for the diapriid *Acanthopria* attacking *Cyphomyrmex minutus* (27% to 53%), although lower than the rates reported for *Cyphomyrmex rimosus* (70%) when attacked by the diapriids *Mimopriella* and *Acanthopria* [18]. In the case of the fungus growing ant *Trachymyrmex* cf. *zeteki*, 15% to 34% of field collected nests were found to be parasitized by diapriid wasps [19]. The frequency of *S. talitae* parasitism in *A. subterraneus* nests was slightly lower than that observed in basal attines such as *Cyphomyrmex* and *Trachymyrmex*. These basal attines construct relatively small nests close to the soil surface, with fewer workers and immature stages when compared to higher attines. Therefore, access to brood chambers by the female parasitoids could be easier, resulting in higher parasitism rates than those seen for *A. subterraneus*.
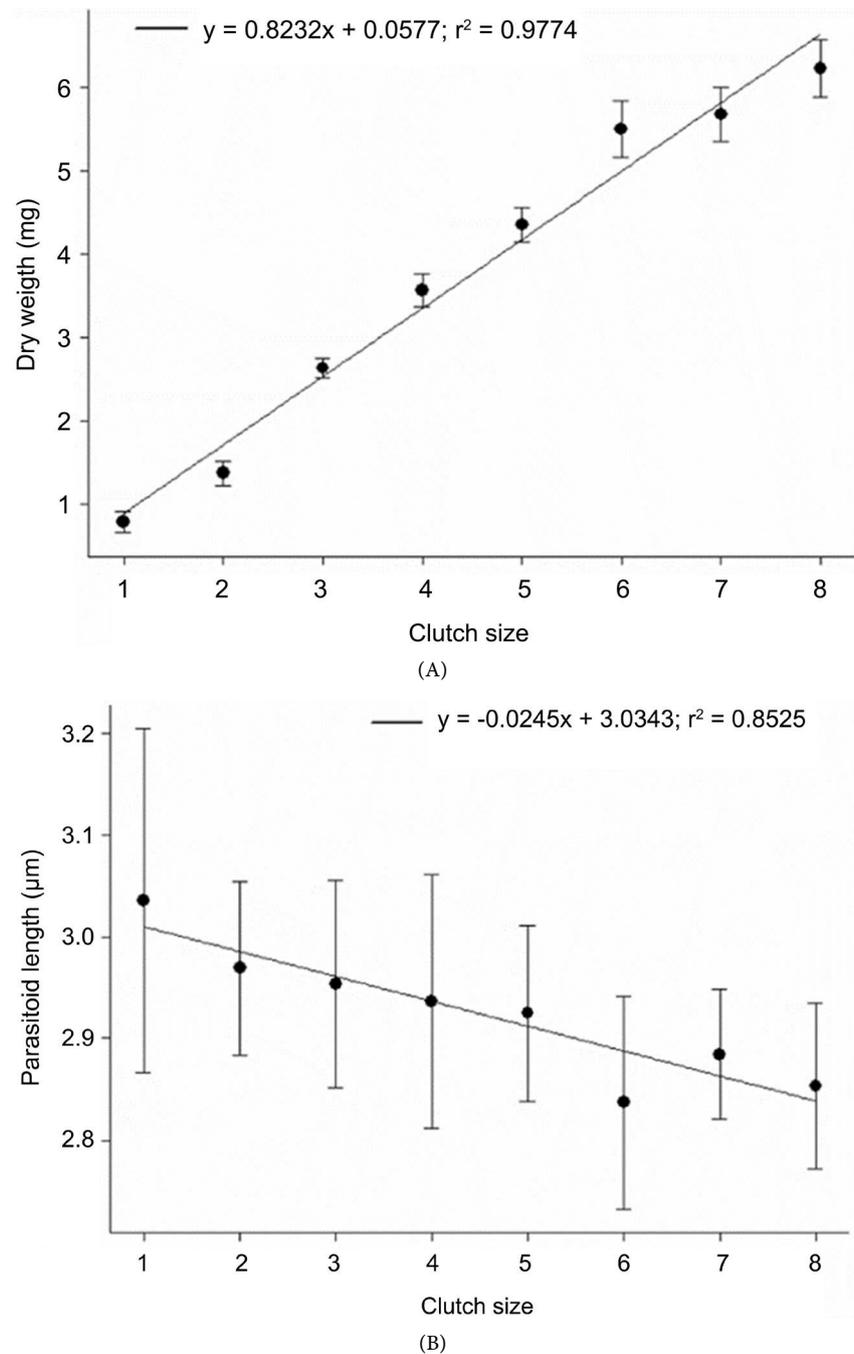
Figure 4. Association between clutch size and dry weight (mg) of parasitized hosts and parasitoid pharate adult size (length μm). (A) Dry weight of parasitized host larvae associated to clutch size. Results were plotted from the dry weights (mg) of individual parasitized host larvae of each clutch size group. Only data for up to eight parasitoids per host larva were shown here; (B) Relationship between pharate adult parasitoid size (length in mm) and clutch size. Results were plotted from the measurements of individual adult *S. talitae* following removal from the host. The regression analysis and r² values are shown as inserts.

When considering the results of the current study for within-colony parasitism by *S. talitae*, on average 60% of the larvae per nest were found to have been

parasitized. This level of parasitism will have a serious knock-on effect on these colonies, resulting in a future shortage of worker ants. All parasitized nests evaluated here did not survive more than 2 - 3 months after collection in the field, whereas most healthy nests had a normal life-span of 2 - 5 years in the laboratory (RIS personal observation).

The within-nest parasitism rate for *S. talitae* recorded here was substantially higher than that previously observed for other diapriid species. In the case of Diapriidae attacking *T.* cf. *zeteki*, the mean rate of larval parasitism per nest was 33.9% [19]. In Panama, the within-nest parasitism of *C. rimosus* by *Acanthopria* was 34%, but in Puerto Rican populations, it was as low as 16% [18]. Data published by Loiácono *et al.* [22], obtained from a single *A. subterraneus* nest, showed that 100% of the larvae had been parasitized by *S. talitae*.

The exact developmental stage at which *S. talitae* attacks *A. subterraneus* larvae has yet to be determined. Many Diapriinae species preferentially parasitize late instar dipteran larvae [24] or the pupal stage [25] [26] [27]. *Acanthopria* and *Mimopriella* have been observed to attack mid- or late-instar larvae of *Cyphomyrmex* spp. (Attini) [18]. *Szelenyiopria pampeana* were found to parasitize late instar *Acromyrmex lobicornis* larvae [28].

Szelenyiopria talitae was shown here to oviposit up to 12 eggs per host and the number of parasitoid larvae developing in each host was positively correlated with host size. This was also the case for the phorid *A. attophilus*, which preferentially parasitized larger worker ants to ensure the progeny have sufficient resources for the successful development of various larvae developing within a single ant host [29]. The data showed that 29% of the hosts had two parasitoids and this was significantly higher than all other frequencies. Solitary parasitism (one parasitoid per host) was observed at an average rate of 14%. Although Loiácono and co-workers [22] stated that solitary parasitism of *A. subterraneus* by *S. talitae* was more frequent than gregarious parasitism, their results were obtained from a single nest. Differences in the frequencies of solitary and gregarious parasitism could be related to the range of sizes of the host larvae on offer. Leaf-cutting ants are highly polymorphic [30] and *Acromyrmex* colonies contain larvae with a range of different sizes [31]. From the data presented in the supplementary material (Table S1), we divided *A. subterraneus* larvae into 6 size classes with lengths varying from 1.4 to 4.9 mm and dry weights ranging from 0.137 to 4.1 mg (Figure S1). The size (length) of parasitized larvae was also evaluated for different numbers of parasitoids per host, but this data is not presented here as the parasitoids cause deformation of the hosts, invalidating this type of measurement. Therefore, we only present data for dry weight in relation to the different numbers of parasitoids per host. The dry weights ranged from 0.8 mg for hosts with a single parasitoid to approximately 6 mg for hosts with 8 parasitoids (Figure 4(A)).

The positive correlation (r^2 = 0.97) seen in our experiments between the number of diapriids per host and host dry weight (Figure 4(A)) indicated that *S. talitae* could differentiate between the different sizes of ant larvae, laying more

eggs in larger larvae. Parasitoids are able to differentiate between different sized hosts, ovipositing larger numbers of eggs in larger hosts. In the case of *Trichogramma minutum* Riley, a lepidopteran endo-parasitoid, larger hosts received more eggs [32]. A preference for larger hosts was also seen for phorids, with, for example, *A. attophilus* parasitizing larger foraging ants, which could accommodate higher numbers of parasitoid larvae per host [12].

It was interesting to observe the correlation between pharate adult size and the number of parasitoids per host, which indicated competition for host resources between siblings. The results showed a negative correlation between pharate adult size (length) and clutch size ($r^2 = 0.85$; **Figure 4(B)**). However, this small difference in size did not result in delayed development of the pharate adults, which emerged from the host over a period of 24 h (data not shown). Similar results were seen for the gregarious parasitoid wasp *Anaphes flavipes* (Hymenoptera: Mymaridae), when the adult body size of the offspring decreased with higher numbers of siblings per host [33].

The study of host-parasitoid interactions in leaf-cutting ants is more complex than that seen in most other insects. Leaf-cutting worker ants are grouped into castes, displaying polymorphism and performing different tasks in the colony, which can influence parasitism rates by phorids [34]. Furthermore, leaf-cutting ant larvae present in the nests have a wide range of sizes, which, as seen here, could influence the number of eggs laid in each host.

5. Conclusion

Although leaf-cutting ants have evolved complex behavioral and chemical defense strategies to protect themselves and the colony from natural enemies, *S. talitae* females are still able to reach the subterranean brood chambers and attack the larvae. The high levels of parasitism observed here indicated that this parasitoid has a deleterious effect on colony health and could act as a natural regulator of ant populations.

Acknowledgements

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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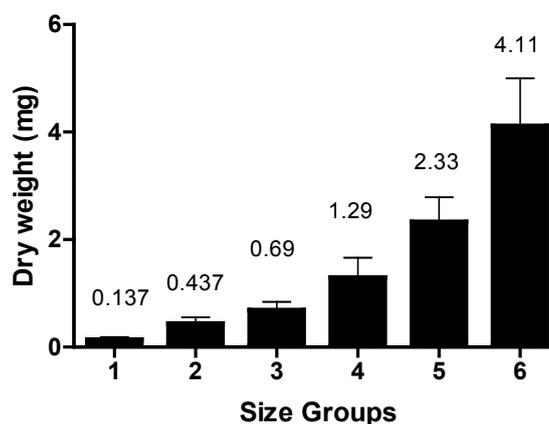
Appendix

Figure S1. Division of healthy *Acromyrmex subterraneus* larvae into 6 weight groups (dry weight in mg). The numbers above the columns are the means and the error bars are standard deviation.

Table S1. Division into size group of *Acromyrmex subterraneus* larvae.

Healthy larvae	
mm	Size Group
up to 1.49	1
1.5 - 1.9	2
2 - 2.49	3
2.5 - 2.9	4
3 - 3.9	5
4 - 4.9	6

Table S2. Number of *Szelenyiopria talitae* parasitoids per host larvae (clutch size) in relation to dry weight (μg) and pharate adult size (length mm).

Parasitoids per host	Dry Weight (μg)			Length (mm)		
	Mean	SD	n	Mean	SD	n
1	0.78	0.24	32	3.04	0.34	48
2	1.36	0.30	32	2.97	0.17	67
3	2.63	0.24	32	2.95	0.20	77
4	3.56	0.40	32	2.94	0.25	57
5	4.35	0.41	15	2.92	0.17	32
6	5.50	0.69	22	2.84	0.21	24
7	5.68	0.65	21	2.88	0.13	13
8	6.23	0.69	14	2.85	0.16	11