Effects of Psychodiella sergenti (Apicomplexa, Eugregarinorida) on Its Natural Host Phlebotomus sergenti (Diptera, Psychodidae)

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ABSTRACT Phlebotomine sand flies (Diptera, Psychodidae) are important vectors of human pathogens. Moreover, they possess monoxenous parasites, including gregarines of the genus Psychodiella Votypka, Lantova, and Volf, which can negatively affect laboratory-reared colonies, and have been considered as potential candidates in biological control. In this study, effects of the gregarine Psychodiella sergenti Lantova, Volf, and Votypka on its natural host Phlebotomus sergenti Parrot were evaluated. The gregarines increased the mortality of immature sand fly stages, and this effect was even more apparent when the infected larvae were reared in more dense conditions. Similarly, the gregarines negatively affected the survival of adult males and females. However, no impact was observed on the mortality of blood-fed females, the proportion of females that laid eggs, and the number of eggs oviposited. The 10-times higher infection dose (50 versus five gregarine oocysts per one sand fly egg) led to ~10 times more gamonts in fourth-instar larvae and two or three times more gamonts in females and males, respectively. Our study clearly shows that Ps. sergenti is harmful to its natural host under laboratory conditions. However, its potential for use in biological control is questionable as a result of several factors, including this parasite’s strict host specificity.

KEY WORDS gregarine, sand fly, mortality, fecundity, infection dose

Phlebotomine sand flies are vectors of important human pathogens such as Leishmania Ross, Bartonella Strong, O’Connor, Winkler, and Steigerwalt, and phleboviruses. They are, however, parasitized by various organisms such as viruses, bacteria, fungi, nematodes, mites, and protists, including gregarines (reviewed by Warburg et al. 1991). Adult sand flies feed on plant sugars; females need blood to acquire nutrients for egg production. Very little is known about the sand fly breeding sites; in general, eggs are laid to the moist soil, animal burrows, caves, or leaf litter, and four larval instars feed on organic detritus (Lane 1993), sometimes also on dead bodies of adults (Adler and Mayrink 1961). The terrestrial development in dark humid sites facilitates growth and persistence of various sand fly entomopathogens, and, at the same time, complicates their collection and examination (Warburg 1991).

Gregarines are parasites of invertebrates, particularly insects. Their effects to hosts vary, and gregarines possessing merogony in their life cycle have even been considered a potential tool for biological control (Perkins et al. 2000). Within the nematoceran Diptera, two gregarine genera have been recently distinguished: the genus Ascogregarina Ward, Levine, and Craig parasitizing mosquitoes and the genus Psychodiella Votypka, Lantova, and Volf parasitizing sand flies (Votypka et al. 2009). Neither genera undergoes merogony, and various authors have come to different conclusions concerning their effects to the hosts (Barrett 1968, Walker et al. 1987, Wu and Tesh 1989, Siegel et al. 1992, Sulaiman 1992, Comiskey et al. 1999).

Psychodiella sergenti Lantova, Volf, and Votypka is a recently described specific pathogen of Phlebotomus sergenti Parrot (Lantova et al. 2010). This sand fly is an important vector of human cutaneous leishmaniasis caused by Leishmania tropica (Wright) (reviewed by Jacobson 2003). Newly hatched sand fly larvae become infected by ingesting gregarine oocysts, each of which contains eight sporozoites. The sporozoites then develop in the larval intestine. In adults, the gamonts are found in the hemocoel, and the sexual development of Ps. sergenti, that is, the formation of syzygy followed by creation of gametocysts with oocysts inside, occurs exclusively in blood-fed females. In gravid females (females that took a blood meal and developed oocytes), gametocysts attach to accessory glands, oocysts are injected into the gland lumen, and during oviposition they adhere to the chorion of eggs (Lantova et al. 2010).

Psychodiella gregarines occurred naturally in a colony we had established from Ph. sergenti collected in Turkey. After several generations, the colony began to suffer from high adult mortality, and dissections revealed hemocoels heavily infected by gamonts and gametocysts. To reduce the intensity of infection and...
increase the fitness of the colony, *Ph. sergenti* eggs were washed by a series of reagents. However, this procedure never completely cleaned the eggs and had to be repeated every generation. The current study, focusing on the effects of *Ps. sergenti* on its natural sand fly host, was made possible only when a new, gregarine-free *Ph. sergenti* colony was established.

**Materials and Methods**

**Sand Flies and Gregarines.** A colony of *Ph. sergenti* free of gregarines was established in 2001 from females originating from Israel (further referred to as IS colony). The infected colony of *Ph. sergenti* used as a source of oocysts was established in 1998 from females originating from Turkey (further referred to as TU colony). The two colonies were kept separately in two insectaries, and maximum care was taken to prevent cross-contamination.

Sand flies were reared at 26°C, in standard conditions used in our laboratory (Volf and Volfova 2011). To sustain the TU colony, it was necessary to reduce gregarine infections by a series of disinfecting solutions described by Poinar and Thomas (1984). Two- to 5-d-old eggs were washed from the rearing pot with distilled water to filter paper in a Büchner funnel connected to a water pump. First, 70% ethanol was used with the water pump on for 10–30 s until all excess liquid was removed. Then, eggs were washed with 5.24% sodium hypochlorite (NaClO) with the pump off for 3.5 min, followed by removing all remaining liquid by turning the pump on for 1 min. Subsequently, 10% sodium thiosulfate (Na2S2O3) was used for 3.5 min with the pump off. Finally, the eggs were thoroughly washed with distilled water with the pump on and washed down into a new clean pot (Poinar and Thomas 1984).

The process of the collection of gregarine oocysts and experimental infection was described by Lantova et al. (2010). In brief, 30 *Ph. sergenti* females (TU colony) were collected after oviposition and homogenized in 500 µl of phosphate-buffered saline. This solution was filtered through gauze and centrifuged (1,700 *g* for 5 min (MiniSpin, Eppendorf AG, Hamburg, Germany); the supernatant was discarded, the pellet was resuspended in 200 µl of water, and the number of oocysts was determined using a Bürker counting chamber and an optical microscope (CX31, Olympus, Tokyo, Japan). The appropriate volume of the oocyst suspension, corresponding to the required number of oocysts, was then resuspended in water to a total volume of 1 ml and sprinkled over and mixed with four small heaps of larval food placed in each rearing pot.

**Effects of Gregarine Infection on Sand Fly Mortality and Fecundity.** To evaluate the effect on immature and adult sand fly stages, two groups of sand flies were established; gravid *Ph. sergenti* females from gregarine-free IS colony were allowed to oviposit, and eggs counted by a stereomicroscope (SZH-ILLD, Olympus Optical, Tokyo, Japan) were placed into rearing pots using a fine brush. In half of the pots, gregarine oocysts corresponding to an infection dose of 50 oocysts per egg were added to the food of first-instar larvae. The remaining pots served as control. All rearing pots had the same size and shape, and, therefore, the total rearing area was the same for the experimental and the control group.

To evaluate the effect on the sand fly immature stages, three experiments were carried out. In the first experiment, we used eight pots with 350 eggs each; four of them were infected with gregarines, and four served as a control. The second experiment evaluated the effect of higher larval density during their development (possible competition for nutrients or space), and five pots had 400 eggs each and the remaining five had 200 eggs each; none of them was experimentally infected. The third experiment evaluated the effect of the gregarine infection together with higher larval density. Five experimental pots had on average 444 eggs that were infected, whereas each of the five control noninfected pots had on average 257 eggs. In each experiment, all pots were placed into the same rearing box to ensure uniform conditions, and emerging adults from both groups were counted every day.

The infection status in the case of the first and the third experiment was determined using an optical microscope after dissection in phosphate-buffered saline under a stereomicroscope. The number of emerged adults was compared with the number of eggs.

To evaluate the mortality of adults, two groups of sand flies were established, as described above, one noninfected and one infected with a dose of 50 gregarine oocysts per egg. In each group, adults that emerged on the same day were placed in a separate cage. The number of dead males and females and their infection status (determined by dissection under a stereomicroscope, followed by observation in an optical microscope) were recorded daily. The number of dead adults of both sexes was compared between the infected and the control group.

To assess the gregarine effect on blood-fed females, two groups of control and infected sand flies were established, as mentioned above. The mortality of blood-fed females was evaluated after females of the same age from both the control and the experimental group were fed on mice, and the number of dead sand flies and the gregarine infection status were recorded daily. The mortality of the experimental and control group was compared. To assess the gregarine effect on the fecundity, other batches of females (from the control and infected group) that emerged at the same day were fed using an anesthetized mouse, and 6 d after the blood meal (i.e., 1 or 2 d after the defecation of blood meal remains), females were separated into glass tubes. The technique originally used for the establishment of sand fly colonies (Killieck-Kendrick and Killick-Kendrick 1991) was adopted. In brief, glass tubes (5 × 1.5 cm) with wet filter paper (4 × 5 cm) inside were closed with gauze and a plastic ring. A small piece of cotton wool with a 50% sucrose solution was provided to females, and all tubes were put into the same rearing box to ensure uniform conditions. The time of laying eggs, the number of eggs, and the gregarine infection of dead females in both groups were recorded daily and compared.
**Effects of Infection Dose on the Intensity of Infection.** Six groups of ≈15 gravid females (IS colony) were placed into six pots to oviposit, and the number of eggs was counted under a stereomicroscope. Gregarine oocysts corresponding to infection doses of either five or 50 oocysts per egg were added to the food of first-instar larvae. Each infection dose was used in three rearing pots. All pots were placed in the same rearing box. Larvae and adults of both sexes were dissected at different time intervals. In addition, 7-d-old females were fed on mice and dissected at various days after the blood meal. In males and females without the blood meal, the number of gamonts plus gametocysts was compared between the two different infection doses.

To separately assess the larval survival depending on rearing density, an experiment comparing the mortality of noninfected larvae in less dense rearing conditions, the difference was compared with the mortality of noninfected larvae in more dense rearing conditions. The mortality of females after a blood meal (Cox’s F test; F = 1.2019, P = 0.0828) (Fig. 1C).

**Infected males** had significantly higher mortality than the control males (Cox’s F test; F = 2.2126, P < 0.05) (Fig. 1A). Similarly, infected females without a blood meal had higher mortality than the females in the control group (Cox’s F test; F = 1.7472, P < 0.05) (Fig. 1B). Conversely, no statistically significant difference was found in the mortality of females after a blood meal (Cox’s F test; F = 1.2019, P = 0.1822) (Fig. 1C).

**Effects of Gregarine Infection on Sand Fly Fecundity.** Two of 22 infected females did not lay eggs (9.1%), and 14 of 52 noninfected females did not lay eggs (26.9%), but this difference was not significant (Fisher exact test; P = 0.1335). T tests did not reveal any significant differences in the number of oviposited eggs between infected and control females (Table 1). The average numbers were 50 ± 6 eggs and 42 ± 5 eggs (±SEM) per infected and control female, respectively. When only ovipositing females were included, infected ones laid 55 ± 6 eggs (±SEM), and 57 ± 5 eggs (±SEM) were laid by noninfected females.

**Effects of Infection Dose on the Intensity of Infection.** The infection dose of 50 oocysts per egg resulted in more intense infections than the infection dose of five oocysts per egg. In the fourth-instar larvae (20 dissected specimens in each group), a 10-times higher infection dose led to ~10 times more gamonts (45:458), and the difference between groups was highly significant. In males and females without a blood meal (15 dissected specimens in each group), different infection doses resulted in less pronounced but still highly significant differences in parasite numbers. Adding together the numbers of gamonts in all adult sand flies of the same sex, the difference between the higher and lower infection dose groups was ≈1:3 in males (309:994) and <1:2 in females (403:757) (Fig. 2A, Table 2).

In blood-fed females (15 dissected specimens in each group), the dose of 50 oocysts per egg led to a
significant increase in the total number of gamonts plus gametocysts in comparison with the dose of five oocysts per egg. Altogether, this increase was \( \times 1.25 \) (134:314) (Table 2, Fig. 2B).

**Discussion**

Our results showed that *Ps. sergenti* infection negatively affects the survival of immature sand fly stages. In mosquitoes, higher larval mortality as a result of infection with *Ascogregarina* species has been recorded, for example, by Barrett (1968) in *Aedes aegypti* (L.) and Garcia et al. (1994) in *Aedes taeniorhynchus* (Wiedemann). Mosquito gregarines develop intracellularly in the larval intestine and have deleterious effect on the midgut cells (Sanders and Poinar 1973). However, *Ps. sergenti* intracellular stages are not known. Therefore, the main cause of higher mortality of immature sand fly stages could be competition for nutrients and energy between gregarines and a sand fly. This competition may become more important in stressful environmental conditions, as was shown in our experiments: the effect on the mortality was more pronounced when the infected larvae were reared in higher density. The higher larval density, when studied separately, did not have significant negative impact on the survival. Similar conclusions were presented in mosquitoes by Comiskey et al. (1999), showing that *Aedes albopictus* (Skuse) larvae and pupae infected with *Ascogregarina taiwanensis* (Lien and Levine) had higher mortality under nutrient-deficient conditions. The joined negative effect of starvation and the gregarine infection was proved also in the black carpet beetle *Attagenus megatoma* (F.): starving eugregarine-infected larvae were losing weight almost twice as rapidly as the noninfected ones (Dunkel and Boush 1969). Similarly, in starving mealworm *Tenebrio molitor* L., the weight loss of eugregarine-infected pupae was larger than in the noninfected ones (Harry 1967).

In adult sand flies, the infection with *Ps. sergenti* significantly decreased the survival of infected males and females without a blood meal (Fig. 1, A and B), whereas the mortality of blood-fed females was not affected (Fig. 1C). This may indicate that more nutritious diet (blood meal) enables infected females to overcome the negative effect of the parasite. Interestingly, sex-dependent differences were observed in adult mortality: in males, higher mortality was detected from the second day, whereas all females, regardless of their infection status, survived until the eleventh day after emergence from pupa and only started to die thereafter (Fig. 1). This could be ad-

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**Table 1. The effect of *Ps. sergenti* infection on the number of oviposited eggs by *Ph. sergenti* blood-fed females**

<table>
<thead>
<tr>
<th>Infection</th>
<th>All females</th>
<th>Ovipositing females only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td>CTR</td>
<td>52</td>
<td>41.7</td>
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<tr>
<td>INF</td>
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<table>
<thead>
<tr>
<th>t value</th>
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<tbody>
<tr>
<td>-0.9278</td>
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<td>0.36</td>
</tr>
</tbody>
</table>

The terms Mean, Median, SD, Min., and Max refer to the number of oviposited eggs; n, refers to the number of females. CTR, noninfected females; INF, infected females.

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**Fig. 2.** Effects of gregarine infection dose on the number of *Ps. sergenti* individuals in various developmental stages of *Ph. sergenti*. Bars represent: (A) the total number of gamonts in 20 individuals of fourth-instar larvae (L4), 15 males, 15 females without a blood meal; (B) the total number of gregarine developmental stages in 15 females blood-fed on mouse. AG, Age of sand flies in days; ID, infection dose in oocysts per egg; BM, days after a blood meal. ***, Highly significant difference (P < 0.01); *, significant difference (P < 0.05) (see Table 2).
Females

Blood-fed females gamonts were found and counted; in blood-fed females (15 specimens) L4 significant effect on tory results; McCray et al. (1970) did not find any effect of obtained similar results. In mosquitoes, studies of the eggs than nonparasitized ones. reported that parasitized females produced 23% fewer garina culicis of Ps. chagasi, on fecundity gave contradic-


effect of the number of ovipositing females nor sand ßy organs (L. Lantova, unpublished data).

Psychodiella bopictus, which severely damages them (Barrett 1968). However, sand fly gregarines of the genus Psychodiella develop in the body cavity of their hosts attached to the oviducts or accessory glands. Histology, however, did not reveal any specific damage to sand fly organs (L. Lantova, unpublished data).

Psychodiella gregarines seem to have no effect on sand fly fecundity, as in our experiments the infection affected neither the number of ovipositing females nor the number of oviposited eggs. In L. longipalpis sand flies infected with Ps. chagasi, Wu and Tesh (1989) obtained similar results. In mosquitoes, studies of the effect of Ascogregarina on fecundity gave contradictory results; McCray et al. (1970) did not find any significant effect on Ae. aegypti infected by Ascogregarina culicis (Ross), whereas Comiskey et al. (1999) reported that parasitized females produced 23% fewer eggs than nonparasitized ones.

Although the gregarine does not affect blood-fed females, Leishmania parasites do, with the effect being significant under stressful conditions (Rogers and Bates 2007). This may raise a question about a possible synergistic effect of both parasites on the sand fly. In nature, however, such coinfections are very rare, mainly because even in leishmaniasis foci the percentage of Ph. sergenti carrying L. tropica is very low (e.g., Volf et al. 2002, Gebre-Michael et al. 2004).

In fourth-instar larvae, the 10-times higher infection dose led to ~10 times more gamonts, whereas, in adults, the intensity of infection achieved by higher infection dose was only two or three times higher in females and males, respectively (Fig. 2A). We suppose that the number of gregarines that can develop in the host is limited, and the pupal stage is clearly the most critical period for gregarine survival. The effect of the infection dose has not yet been studied in sand fly gregarines, and comparative data are available only from one mosquito experiment: Reyes-Villanueva et al. (2003) exposed larvae of Ae. aegypti and Ae. albopictus to various oocyst concentrations of As. culicis and As. taivanensis and found linear dependence of infection intensity on the infection dose.

Dissections confirmed our previous observations on the life cycle of Ps. sergenti (Lantova et al. 2010), that is, the gregarines were able to complete the life cycle exclusively in females after a blood meal. Only gamonts were present in males and females without a blood meal. Importantly, it was shown that even the lower dose of five oocysts per egg was sufficient for successful infection and completing the life cycle.

To determine the number of oocysts commonly produced in one laboratory-reared Ps. sergenti female from Turkey, the number of oocysts in females 7 d after a blood meal was counted using a Bürker counting chamber. Average number of oocysts per Ps. sergenti female was 15.158 ± 1.807 (±SEM). As this sand fly colony produces 32 eggs per female on average (Dvorak et al. 2006), we could estimate the theoretical infection dose in laboratory conditions as 474 ± 56 gregarine oocysts per sand fly egg. However, not all the oocysts are discharged during oviposition; some of them remain in the body carcasses of dead females, which are collected from the breeding pots after oviposition. The experimental dose used in our study (five or 50 oocysts per egg) is much lower than the one estimated above, but it probably better reflects natural infections in sand fly breeding sites.

Several authors have discussed the possibility of mosquito and sand fly gregarines being useful in biological control with contradictory conclusions; Barrett (1968) and Sulaiman (1992) consider these gregarines useful, although others, for example, Walker et al. (1987), Wu and Tesh (1989), Siegel et al. (1992), and Tseng (2007), do not. Our experiments showed that Ps. sergenti is harmful to its host Ph. sergenti under laboratory conditions, and the effects can be influenced by environmental factors. The gregarine’s potential for use in biological control is, however, limited by its high host specificity (Lantova et al. 2010) and the lack of knowledge about sand fly breeding sites.

Table 2. Statistical comparison of the effects of Ps. sergenti infection dose (5 and 50 oocysts per egg) on the number of gregarines in Ph. sergenti

<table>
<thead>
<tr>
<th>Stage</th>
<th>r value</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>L4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malesa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>−2.5880</td>
<td>25</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4</td>
<td>−2.9426</td>
<td>25</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>7</td>
<td>−1.9848</td>
<td>25</td>
<td>0.0570</td>
</tr>
<tr>
<td>9</td>
<td>−2.9449</td>
<td>25</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>10</td>
<td>−3.2516</td>
<td>25</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>13</td>
<td>−2.8811</td>
<td>25</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Femalesa</td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>−3.3093</td>
<td>25</td>
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</tr>
<tr>
<td>4</td>
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</tr>
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<td>9</td>
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<tr>
<td>13</td>
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<td>Blood-fed femalesb</td>
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<tr>
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<tr>
<td>7</td>
<td>−1.9899</td>
<td>25</td>
<td>0.0564</td>
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</table>

In males and unfed females (15 specimens of each stage), only gamonts were found and counted; in blood-fed females (15 specimens of each stage), gamonts and gametocytes were counted and added together. L4, fourth instar larvae (20 specimens).

a Numbers represent age of adult sand flies (days after emergence).

b Numbers represent days after a blood meal.

In males and unfed females (15 specimens of each stage), only gamonts were found and counted; in blood-fed females (15 specimens of each stage), gamonts and gametocytes were counted and added together. L4, fourth instar larvae (20 specimens).

a Numbers represent age of adult sand flies (days after emergence).

b Numbers represent days after a blood meal.
Acknowledgments

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