Mating Flight Initiation and Nutritional Status (Protein and Lipid) of *Solenopsis invicta* (Hymenoptera: Formicidae) Alates Infected with *Thelohania solenopsae* (Microsporidia: Thelohaniidae)

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ABSTRACT Female alates of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), infected with *Thelohania solenopsae* Knell, Allen, and Hazard (Microsporidia: Thelohaniidae) were found to be significantly less likely to be among the early alates to initiate a mating flight. If a flight was initiated, the infected females were significantly more likely to initiate the flight later in comparison with uninfected alates. In addition, many infected female alates seemed not to fly as a significantly greater proportion of the remaining females. Furthermore, infected females exhibited a significantly reduced body weight and a significantly reduced lipid titer, in comparison with uninfected individuals. However, there was no difference between the infected and uninfected alates in the amount of soluble protein. These results suggest that the low-weight, late flying-infected females may be less likely to reach a male mating swarm and less likely to outcompete uninfected females in mating and dispersing. Last, if mated or not, upon landing, the infected females may less likely have the needed energy to initiate a colony.

KEY WORDS microbial control, imported fire ant, pathogen, infection, alate

Red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), has become a serious pest in the southern United States (Vinson 1997). Although pesticides have been used for a number of years to attempt to manage this pest, there has been an interest in finding and effectively using biological control agents (Jouvenaz 1990). Among the organisms of interest are the Microsporidia (Keeling and McFadden 1998). The microsporidian *Thelohania solenopsae* Knell, Allen and Hazard is a fire ant pathogen (Becnel and Andreadis 1999) first isolated from *S. invicta* from Brazil (Allen and Buren 1974). It was described by Knell et al. (1977) and first found in the United States in 1996 (Williams et al. 1998). The pathogen has been reported to reduce the fecundity of queens (Williams et al. 1999), and the colonies seemed to slowly degrade, as evidenced by decreases in brood numbers (Oi and Williams 2002), mound volume (Briano et al. 1995, Cook 2002), and colony death (Knell et al. 1977, Oi and Williams 2002). Vegetative stages of the disease were found in all stages of the ant, although spores were only found in late pupal stages, adult workers, reproductive females, and males (Williams et al. 1999, Oi and Williams 2001). Microsporidia are generally vertically transmitted (Dunn and Smith 2001), and this seems to occur with *T. solenopsae* (Valles et al. 2002). However, horizontal transmission of Microsporidia is known to occur in other insects (Andreadis 1987), and this method of transmission seems to occur in the fire ant, as evidenced by infecting uninfected colonies by introducing brood and workers from a *Thelohania*-diseased colony (Williams et al. 1999, Oi et al. 2001). Furthermore, Chen et al. (2004) has shown the presence of spores in the fecal excretions of workers and in the meconium of pupating larvae, suggesting a possible source of spores that could be spread horizontally. Oi and Williams (2003) showed that infected queens initiated small-infected colonies that were raided by other uninfected colonies; these raiding colonies then became infected. These results suggested brood raiding (Tschinkel 1992, Balas and Adams 1997) as one pathway for horizontal transmission. But there may be others, such as through mating flights that occur with the polygyne as well as the monogyne form (Bhatkar 1990), but there is no information regarding flights by infected queens until now.

First, we investigated the effect of queen infection status on her likelihood to fly early or late during the mating flight. Second, we wanted to know whether there were any differences in the nutritional resources that were carried from the parent colony between infected and uninfected females, because these queens are claustral colony founders and parental resources are essential (Hölldobler and Wilson 1990). Of the several nutritional sources, glycogen is important in a mating flight as a flight fuel (Vogt et al. 2000). In this study, however, we considered the protein and lipid concentrations—the two essential components

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used in the colony foundation and success of claustrally founding ant species such as, *Crematogaster opuntiae, Camponotus festinatus* (Wheeler and Buck 1996), and *Solenopsis invicta* (Buren) (Toom et al. 1976). In the former two, large amounts of protein and lipid accumulate in the gynes at the time of eclosion and mating, and during colony founding, protein is depleted from both the thorax and abdomen and lipid from the abdomen (Wheeler and Buck 1996).

Materials and Methods

Collection of Female Alates during Flight Initiation. We first collected samples of *S. invicta* workers from a number of sites to determine the location of infected colonies. These samples were collected by inserting a 50-ml plastic centrifuge tube that had the inner rim of the tube dusted with talc to prevent ant escape, into the center of the mound. Vials were capped after 1 min, and the ants were then frozen. To determine the infection status, 50–100 worker ants were homogenized in 1 to 2 ml of water in a glass tissue grinder (Pyrex 7725-16). A drop of the resulting solution was examined under 400× magnification with a Nikon Alphaphot-2 light microscope for the presence of *T. solenopsae* spores. Furthermore, a staining protocol by Didier et al. (1995) was used, where in samples were stained with calcafluor white (Polysciences, Warrington, PA) to confirm that the spores were in fact microsporidia.

With this initial survey, we marked five collection sites in central Texas, specifically Brazos County that had mostly *T. solenopsae*-infected *S. invicta* colonies. We then monitored and collected female alates of *S. invicta* (winged virgin reproductive) initiating mating flights at each of these sites beginning from 2001 to 2003 for our experiments. These collections made over a 2-yr period every spring and fall served as replications in our experiments.

Before a predicted mating flight (Markin et al. 1971), we randomly selected a collection site and monitored female alates that were leaving a particular mound to initiate a mating flight. At this point, we began collecting as many female alates as possible from the top of the mounds and associated vegetation by using an insect aspirator. Collections were continued for 10–15 min and designated as the first collection. A second collection was initiated 15–25 min after the first collection was completed, and, depending on the number of female alates that attempted to fly, the collection was conducted for another 15–25 min. These times were chosen based on the experience with the Texas populations (Bhatkar 1990; S.B.V., unpublished data) where many of the flights from polygyne colonies last about an hour. After the nuptial flight had ended, we also collected female alates that did not leave for the nuptial flight by removing dirt from the mound and aspirating as many of the remaining female alates as possible. All samples were placed in 25-ml plastic vials, capped, and labeled as collected. We placed all these vials in a cooler and returned to the laboratory. At the time of collecting female alates, workers also were collected from each colony by inserting a 25-ml plastic vial into the colony until it was half full and capped. The workers were collected just to ensure that the colony from which we collected female alates was in fact infected.

Infection status and body weight. Both the workers and female alates, once brought to the laboratory were examined for infection by using slightly different procedures. To examine infection in workers, the workers were homogenized immediately in 1 to 2 ml of water in a glass tissue grinder and a drop of the resulting solution was examined for infection as described above. The female alates, however, collected from either an early flight, late flight, or remaining were killed by freezing and held at −5°C until further analysis. Before the analysis, we removed the vials containing these female alates from the freezer and allowed them to come to room temperature, which took 3 h. This procedure provided a fresh wet weight required for the subsequent analysis. We weighed each female alate individually on an electronic balance to the nearest 0.01 μg (ER-182A, A&D Company, Broomfield, CO). After the determination of each female alate's weight, her infection status was determined as infected or uninfected by following the procedure described above for female alates. Based on the overall collection of early flying, late-flying, and remaining female alates, we also calculated the percentage of infected queens at each site separately. The idea here was to use this information to choose the sites that had approximately the same level of infection so as to reduce the variability between the sites in our experiments, and most importantly to avoid confounding results. All the collection sites were classified as highly infected (>60%), moderately infected (30–60%), or less infected (below 30%) sites. Four of the five sites were moderately infected with 30–60% infection except for one site, which was highly infected. Thus, we disregarded the numbers from that particular site that was highly infected from further analysis.

To evaluate the protein or lipid concentration of the female alates, 60 infected and 60 uninfected female alates collected from the different sites and frozen, but not used in the studies described above, were thawed and weighed as mentioned above. They were then subjected to two different bioassays as follows.

Protein Assay. We used both infected as well as the uninfected homogenate samples (prepared as described above) for the protein assay. The assay was performed according to Wheeler and Buck (1996) by using a bicinchoninic acid assay kit purchased from Pierce Chemical (Rockford, IL) with two slight modifications, mainly because of the need to determine the infection status of the female alates. One modification was the addition of 250 μl of distilled water to allow us to macerate the tissue, and the second modification was the removal of 15 μl of the macerated tissue to allow us to determine the presence or absence of microsporidia infection. The first modification necessitated the development of a new standard curve (see below); the second modification required a calculation to add back the small removed sample.

Table 1. Infection of alate females during and just after a mating flight from four sites and total for all sites (bottom row)

Site	First collection[a]			Second collection[b]			Alates remaining in the nest		
	Total no. ants	No. infect.	% infect.	Total no. ants	no. infect.	% infect.	Total no. ants	No. infect.	% infect.
1	110	32	29.1	96	52	54.2	99	82	82.8
2	60	10	16.7	52	10	19.2	51	31	60.2
3	60	8	13.3	105	35	33.3	60	26	43.3
4	38	7	18.4	11	6	54.5	12	8	66.7
Total	268	57	21.3	264	103	49.2	222	147	66.2

No. infect. is number infected with *Thelohania*, and % infect. is percent infected with *Thelohania*.
[a] First collection is females collected for the first 10–15 min after the initiation of a mating flight.
[b] Second collection is females collected 15–25 min after the first collection for 15 min.

After determining infection status, 1 ml of 1 N sodium hydroxide was added to the female alate homogenate in the tissue grinder, and the solution was mixed. From the tissue grinder a 1-ml subsample of the preparation was removed and pipetted into a 16- by 13-mm glass test tube. The bicinchoninic acid reagent was added to the test tube in the amount of 1 ml, and the test tubes were heated at 60°C for 60 min in a dry bath incubator (Fisher, Pittsburgh, PA). After the solution had cooled to room temperature, the samples were read in a DU-7 spectrophotometer (Beckman Coulter Fullerton, CA) at 562 nm by using a NaOH blank. The readings were compared with a standard protein titer curve generated using a range of concentrations of bovine albumin in 1 N NaOH plus the 250 μl of distilled water that was added to facilitate maceration. After the raw protein concentrations were determined for the 1-ml subsample, calculations were made based on the original total sample (accounting for the removed 15 μl), so that the data could be expressed as a percentage of the alates' total body weight. The protein concentrations were divided into the total body weight for each female to yield data in micrograms as a proportion of the female's body weight.

Lipid Assay. As for the protein assay, we used infected as well as the uninfected homogenate samples (prepared as described above) for the lipid assay. This assay was performed according to Van Handel (1985) with a modification to allow us to determine the infection status (removal of 15 μl of the homogenate), and by the addition of the 250 μl of distilled water. Then, 0.5 ml of a 1:1 mixture of chloroform and methanol was added to the tissue grinder and mixed. The sample was then carefully poured into a 16- by 13-mm glass test tube. Thereafter, the assay followed the procedure of Van Handel (1985).

Test tubes with the ant homogenate and 0.5 ml of chloroform–methanol were heated at 100 + 5°C until the solvent had evaporated. Tubes were allowed to cool to room temperature, and then 0.2 ml of 85% sulfuric acid was added to each tube. Tubes were agitated to mix the contents and heated at 100 + 5°C for 10 min. After the tubes had cooled to room temperature, 4.8 ml of vanillin-phosphoric acid reagent was added, mixed, and the tubes were held for 5 min to allow the color to develop. The samples were read in a spectrophotometer at 525 nm by using a blank of the vanillin-phosphoric acid reagent.

Lipid concentrations were determined from a standard line generated by using a series of concentrations of a commercially available Wesson pure vegetable oil in chloroform. These samples were treated as the ant samples, i.e., the addition of 250 μl of distilled water. After the raw concentrations were determined, calculations were made to add an equivalent amount represented by the removal of 15 μl used to determine the infection status. The total lipid concentration data were divided into the females total body weight to give the data in micrograms as a proportion of body weight.

Voucher Information. Vouchers of the ants used in these studies were placed in the Texas A&M University Insect Collection, College Station, TX.

Statistical Analysis. We performed a two-way analysis of variance (ANOVA) on the number of infected female alates that initiated the mating flight as a dependent variable, with collection sites and time of flight (early, late, and remaining) as two independent variables. The interaction between the two factors also was included in the model. If the collection sites and time of flight had a significant effect on the number of infected female alates that initiated the mating flight, a multiple comparison analysis was done using Tukey's honestly significant difference (HSD) mean separation technique.

We performed independent sample *t*-tests to detect differences between the infected and uninfected female alates in their body weight and the mean amount of lipid and protein quantified as percentage of body weight from the respective female alates. The statistical analyses were performed using SPSS Statistical Software version 10.1 for Windows and JMP7 Statistical Discovery Software version 4.0.4 (SAS Institute, Cary, NC).

Results

Mating Flight. The data on the total number of female alates at the initiation of a mating flight collected from four different sites and at two different time periods and those remaining are shown in Table 1. The table also provides information on the percentage of infected females collected from each site.

The results from the two-way ANOVA (Table 2) suggest that there is no significant effect of the collection sites on the number of female alates that initiate flight; however, the time at which the collections were made had a significant effect. A further multiple-

Table 2. Two-way ANOVA on the main effects of collection time and site on the percentage of female alates infected with *Thelohania*, during and just after a mating flight from four sites in experiments and a comparison of the collection times tested by Tukey's HSD multiple comparison tests

	df	F ratio	Prob > *F*
Source			
Collection time	2	22.54	0.0016
Site	3	4.01	0.056
Collection time * site	6	0.08	0.90
Factor and levels		Mean ± SE	
Time			
First collection		19.37 ± 4.61a	
Second collection		40.30 ± 4.60b	
Remaining		63.25 ± 4.61c	

Mean numbers represent the percentage of infected female alates. Mean percent followed by the same letter are not significantly different at $P < 0.05$.

comparison test revealed that difference is significant between the first and the second collection time and also from those that were remaining in the nest. Also, the percentage of infected females collected, increased from the first collection time to the second.

Total Body Weight. The weights of all infected and uninfected females collected from mating flights were combined and used to determine the protein and lipid data. The effects of infection on the female alates' mean body weight from this data set showed that uninfected females were significantly heavier than infected females ($t = -2.11$, df = 118, $P = 0.037$) (Table 3).

Protein Assay. Data from the protein assay are shown in Table 4. The total mean concentration of protein from infected and uninfected female alates collected during the mating flight was not significantly different, nor was there any significant difference when the protein was expressed as a percentage of the female's body weight ($t = 1.04$, df = 58, $P = 0.16$).

Lipid Assay. Data from the lipid assay are shown in Table 5. There is a significant difference in the amount of total lipid extracted from infected and uninfected female alates collected during the mating flight as well as a significant difference when the lipid is expressed as a percentage of the female's body weight ($t = 3.24$, df = 58, $P < 0.001$).

Discussion

The results show that <20% of the female alates that initiated an early mating flight were infected with *T. solenopsae*. In contrast, females that initiated their mating flight later or did not attempt to initiate a mating flight were more likely to be infected, 40 and 63%, respectively. These results suggest that *Thelohania*-infected female alates are slower to initiate a mating flight and that many do not attempt to fly.

The weights of infected female alates were found to be significantly less than those of uninfected alates. These data are in agreement with Cook et al. (2003), who reported that infected alates usually weighed less. Toom et al. (1976) reported on the importance of queen body reserves to queens as they embark on a mating flight and the foundation of new colonies. Such reserves are essential to egg laying and nanitic brood development (Toom et al. 1976). Vander Meer et al. (1992) also reported that smaller queens lay fewer eggs and care for fewer larvae. As a result, these founding colonies are more likely to be susceptible to predators and usurpation by other nearby fire ant colonies, further increasing the possibility that these incipient colonies would be usurped, thus providing an additional means of spreading the pathogen.

Although queen weights differed, there was no significant difference in the total extractable protein from infected or uninfected female alates. (It should be noted that >50% of the alates' weight is not extractable as lipid or protein and probably consists of structural proteins that are not available for brood rearing.) However, when looking at the protein titer range, the uninfected females exhibited a much greater variability. When the low body weight of the infected females was taken into consideration, the protein levels were higher in the infected alates. Although this higher protein titer is associated with the *T. solenopsae* infection, there are several explanations for these data. The effects of *T. solenopsae* on the infected females lipid titer provide the most likely explanation. The lipid levels were significantly lower in the infected alates based both on the total lipid levels, and as a percentage of the female's weight.

Table 3. Mean weight of uninfected and infected female alates collected from mounds during and after the mating flight

Infection status	No. female alates	Mean wt (μg)
Uninfected	60	9,696.66 ± 263.62a
Infected	60	8,971.66 ± 220.29b

Means followed by same letter are not significantly different ($t = -2.11$, df = 118, $P = 0.037$).

Table 4. Protein concentration of female alates in micrograms and as a percentage of the females' body weight, and a comparison of protein concentration in infected and uninfected female alates made by *t*-test ($t = 1.04$, df = 58, $P = 0.16$)

Infection status	No. of female alates	Protein (μg)	% protein by body wt
Infected	30	2193.33 + 59.48	24.21 + 0.72a
Uninfected	30	2166.33 + 76.58	22.63 + 0.87a

Means followed by same letters are not significantly different.

Table 5. Lipid concentration of female alates in micrograms and as a percentage of the females' body weight, and a comparison of lipid concentration in infected and uninfected female alates made by *t*-test ($t = 3.24$, df = 58, $P < 0.001$)

Infection status	No. of female alate	Lipid (μg)	% lipid by body wt
Infected	30	1072.83 + 66.88	10.69 + 0.68a
Uninfected	30	1353.97 + 53.83	13.98 + 0.49b

Means followed by same letters are not significantly different.

Thus, the lower weight of infected females is most likely because of a low lipid titer. As a result, the proteins are the major contributor to the weight of the infected queens, as the protein data show (assuming the structural proteins are not impacted by the infection). The low lipid levels of infected queens also would suggest that these queens would have less resource available to found a viable colony.

In conclusion, the data reported here suggest that infected queens weigh less, have less lipid reserves, and fly later in the mating flight, which in turn suggests, that the infected queen may be less likely to find a mate and thus may be more likely to be virgins upon landing and initiating a colony. Established fire ant colonies are known to raid these newly founded virgin queen colonies where the male larvae are removed and the queens are abandoned or adopted. As a result, the male larvae, or in some cases the male-producing queens, could end up in established polygyne colonies. We know that some virgin dealate females end up in polygyne colonies and lay haploid eggs producing male larvae (Bashir et al. 2003), whereas others produce diploid males (Hung et al. 1974). Furthermore, because these male larvae, particularly the diploid males, are more likely to be killed (Ratnieks 1990, Bernasconi and Keller 1996), the result is that they serve as food for other larvae (Tschinkel 1993). If these diploid male larvae are from infected virgin queens and are infected with *T. solenopsae*, then this would be one way to transmit the infection to other larvae within an uninfected colony. Thus, the raid of the virgin queen colonies by other fire ant colonies, and its subsequent cannibalism (Ratnieks 1990, Tschinkel 1993) would further support the suggestion by Oi and Williams (2003), on the impact of brood raiding as a means of horizontal transmission. This information could have important implications in the control of fire ants.

Lastly, whether or not infected males fly was not investigated in this study, because the ability of an infected male in successfully transferring the infection to a female during mating flight is unknown. Once this question is addressed, the impact of infected males on the horizontal transmission of the pathogen can be answered.

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