

Prevalence of *Thelohania solenopsae* infected *Solenopsis invicta* newly mated queens within areas of differing social form distributions

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Abstract

Newly mated queens (NMQs) originating from monogyne red imported fire ant (*Solenopsis invicta*) colonies and following a mating flight, initiate new colonies by sealing themselves in a nuptial chamber and relying solely on their own fat and crop reserves, as well as no longer needed wing muscles to rear their first workers (claustral colony foundation). This method of colony-founding is rarely successful for polygyne-derived NMQs, whose low weight critically limits the number of first workers they are able to produce. However, this observation may be confounded by the parasitic microsporidium, *Thelohania solenopsae*, thus far found to persist only in association with polygyne colonies. Infections of this microsporidium reduce the weight of female alates and may explain why polygyne NMQs are unlikely to successfully found colonies claustrally. NMQs collected following mating flights in Gainesville and Ocala, Florida were sorted by weight, checked for insemination and *T. solenopsae* infection. Insemination levels were greater than 90% for all weight classes at both collection sites and were not related to infection. Infection levels were lower in Gainesville than Ocala, averaging 1.67% and 14.14%, respectively. Polygyne-derived NMQs collected in Ocala, defined here as weighing ≤ 12 mg (social form correctly assigned in 85% of samples examined by PCR), had the highest infection levels, 25.37% (17/67) in 2003 and 21.43% (6/28) in 2004. We conclude that infection by *T. solenopsae* cannot be completely responsible for the inability of polygyne NMQs to claustrally establish colonies.

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1. Introduction

Solenopsis invicta Buren (Hymenoptera: Formicidae) female and male alates take flight when environmental conditions are appropriate (Markin et al., 1971), and mate about 300 m in the air. The newly mated queens (NMQs) then land, break off their no longer needed wings and search for a suitable place to dig a nuptial chamber. The NMQs seal themselves in their nuptial chambers (claustral colony foundation) and produce their first set of workers (nantic workers) using only their body reserves, crop contents, and histolysis products from their superfluous wing

muscles. Nantic workers eclose after ca. 30 days and are the smallest workers produced by the queen. These workers then perform all colony functions, including foraging, feeding, and grooming the queen and tending brood. The number of nantic workers produced by a NMQ is critical to successful colony foundation (Porter and Tschinkel, 1986). Therefore, colony-founding success is highly dependent on the amount of initial energy reserves of the founding queen.

Monogyne and polygyne queens can be distinguished by their genotype at the biallelic locus *Gp-9* (Krieger and Ross, 2002). Monogyne queens are always BB and polygyne queens are almost invariably Bb (Krieger and Ross, 2002). Polygyne female alate (Bb), NMQ (Bb), and established polygyne queen weights have been characterized as significantly less than their monogyne counterparts (Vander Meer et al., 1992; Keller and Ross, 1995), even though fixed

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morphometric measures are indistinguishable (Porter, 1992). Therefore the decreased weight of the Bb genotype polygyne NMQ directly impacts its ability to successfully found colonies claustrally (Porter et al., 1988; Keller and Ross, 1993, 1995). It follows then that the ability of polygyne NMQs to initiate colonies claustrally may be reduced even further by infections with naturally occurring or introduced biological control agents. *Thelohania solenopsae* (Microsporidia: Thelohaniidae) infections have been reported to result in low weight female alates (Cook et al., 2003) from an area of Texas with predominantly the polygyne social form (supported by low NMQ insemination rates: ca. 67%). Thus, infected polygyne NMQs would be expected to be less able to found colonies claustrally than uninfected polygyne NMQs.

The release of natural enemies of *S. invicta*, such as a decapitating phorid fly, *Pseudacteon tricuspis* (Diptera: Phoridae), or endoparasites, e.g. *T. solenopsae* (Williams et al., 2003; Pereira, 2003) is part of an on-going biological control program (USDA and others, e.g. see Gilbert and Patrock, 2002) aimed at applying similar population pressures to the imported fire ants as found in their native South America. *T. solenopsae* was first discovered by Allen and Buren (1974) from *S. invicta* workers collected in Brazil. The microsporidian was not found in U.S. populations in the mid-1970s (Jouvenaz et al., 1977); however, two decades later Williams et al. (1998) found it naturally occurring in polygyne populations of *S. invicta* in the United States. Phorid fly parasites of fire ants have been successfully released in many areas of the southeastern United States (Porter et al., 2004). These biological control agents can have confounding effects on unrelated experimental designs using field-collected specimens and introduce factors that must be considered in the analysis of results.

Among collections of NMQs, it is possible to collect infected but inseminated NMQs. Unknown use of these NMQs in experiments not involving *T. solenopsae* could lead to erroneous conclusions and confounded experiments. The potential impact of *T. solenopsae* infected NMQs on unrelated experiments can be addressed if the NMQ prevalence rates are known for the two social forms. We present the weight distributions, as well as insemination and *T. solenopsis* prevalence rates for NMQs collected from Gainesville (predominantly monogyne) and Ocala (predominantly polygyne), Florida field sites in 2003 and 2004.

2. Materials and methods

2.1. Collection of NMQs

Fire ant mating flights can occur any time of the year in north central Florida if environmental conditions are suitable. Flights typically occur the day after precipitation and if there is low wind and warm temperatures (Markin et al., 1971). The largest mating flights occur in late May through June after a buildup of reproductives in the spring. To

obtain sufficient numbers of NMQs, we collected NMQs as they landed on large, paved parking lots, since NMQs are very difficult to locate once they land in vegetation. Newly mated queens were collected from two study sites, Ocala, Marion County, Florida on June 5, 2003 (287 NMQs) and June 3, 2004 (143 NMQs), and Gainesville, Alachua County, Florida on June 21, 2003 (490 NMQs) and May 1, 2004 (510 NMQs). Immediately after returning to the laboratory, the live NMQs were weighed (OHaus GA200-D, OHaus Corp, Florham Park, NJ) and each collection was independently grouped into the following size classes: <10, 10 < 12, 12 < 13, 13 < 14, and ≥ 14 mg. Samples were stored in 100% ethanol in the freezer until analysis. Both collection sites are a mosaic of monogyne and polygyne colonies; however, the Ocala site has a much higher proportion of polygyne colonies than the Gainesville site (Fritz and Vander Meer, 2003; Porter, 1992). Thus, the Ocala site presents a higher probability of collecting large numbers of polygyne colony produced NMQs.

2.2. Determination of social form

Monogyne and polygyne NMQs exhibit a bimodal weight distribution, with polygyne NMQs weighing significantly less than monogyne NMQs (Keller and Ross, 1999; DeHeer, 2002). Established monogyne and polygyne queens can be distinguished by their genotype at the biallelic locus *Gp-9* (Krieger and Ross, 2002). Monogyne queens are always BB and although polygyne-derived female alates can be BB, Bb, and/or bb (Krieger and Ross, 2002), all functional polygyne queens are *Gp-9^{Bb}*. *Gp-9^{BB}* NMQs are executed if they attempt to enter monogyne or polygyne colonies and *Gp-9^{bb}* individuals are thought to perish as brood or young adults (Ross, 1997; Ross and Keller, 1998); therefore, functional *Gp-9^{BB}* and *Gp-9^{bb}* polygyne-derived queens have not been found. A sample of NMQs from each site and weight class was genotyped for the *Gp-9* locus to confirm the weight method of assigning social form. NMQs were maintained in 100% ethanol until DNA isolation of the head and thorax using the GNOME[®] DNA Isolation Kit (Qbiogene, Inc., Carlsbad, CA). Samples were genotyped for the *Gp-9* locus by PCR amplification and subsequent restriction enzyme digestion with *Bsa*AI (New England BioLabs Inc.) according to the protocols of Krieger and Ross (2002). For our experiments, collected NMQs that weighed ≥ 14 mg are considered monogyne while those that weighed ≤ 12 mg are considered polygyne (Keller and Ross, 1999; DeHeer, 2002).

2.3. NMQ insemination rates

Insemination of *S. invicta* NMQs was determined by removing their spermatheca by gently pulling on the sting. The spermatheca was visually examined using a dissecting microscope to determine if it contained sperm. Inseminated queens have an opaque, pearl-white,

Table 1
Distribution of newly mated queens (NMJs) among different weight classes

Weight class (mg)	Gainesville		Ocala	
	2003	2004	2003	2004
<10	3 (1)	0 (0)	20 (7)	7 (5)
10 < 12	13 (3)	12 (2)	47 (16)	21 (15)
12 < 13	23 (4)	12 (2)	25 (9)	11 (8)
13 < 14	83 (17)	53 (10)	44 (15)	27 (19)
≥ 14	368 (75)	433 (86)	151 (53)	77 (53)
Total NMJs	490	510	287	143

Percentages for each weight class are shown in parentheses. Total number of NMJs collected and sample size for NMJ insemination prevalence and *S. thelohania* infection prevalence in NMJs are the same except for the ≥ 14 mg weight class where a random sub-sample (Gainesville: 69 and 36; Ocala: 65 and 35 in 2003 and 2004, respectively) was used because of the large number of NMJs in this weight class. Newly mated queens were collected immediately following large spring mating flights at two sites, Gainesville (predominately monogyne) and Ocala (predominately polygyne), in 2003 and 2004.

kidney-shaped spermatheca while uninseminated queens have an empty, transparent spermatheca. From the stored NMJ collections, we examined all NMJs from each weight class; however, due to the large number of samples collected in the ≥ 14 mg weight class, a subset of the NMJs was randomly selected and analyzed for insemination. See Table 1 for sample size examined for each weight class and location.

2.4. *Thelohania* prevalence rates

After checking the spermathecae for insemination, the abdomens of the NMJs were placed individually into 1.7-mL microcentrifuge tubes and macerated in a small amount of water using disposable plastic pestles. Aliquots of the resulting extracts were placed individually on glass slides and examined at 400× magnification on phase three mode for the presence of *T. solenopsae* using a phase contrast microscope (Nikon Labphot-2, Melville, NY; Oi and Williams, 2002, 2003). Additional *T. solenopsae* infections may have been detected using a more sensitive PCR method, Valles et al., 2002. Due to the large number of samples collected in the ≥ 14 mg weight class, a randomly selected subset of the NMJs was analyzed for *T. solenopsae* infection. See Table 1 for the sample size examined for each weight class and location.

2.5. Statistical analyses

The NMJ weights were not normally distributed and were therefore compared using the Wilcoxon Rank Sums Test to examine differences in weight distributions between sites for the same year and within each site between years. Contingency analyses were used to examine differences between insemination and infection rates at these two sites. All statistical analyses were performed using JMP 5.0 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Weight distributions of NMJs from two field sites over two years

The NMJ weight distributions between each site were significantly different (Fig. 1). Newly mated queens collected in Gainesville were significantly heavier than those collected in Ocala in both 2003 [mean (mg) ± SEM: Gainesville 14.55 ± 0.05, Ocala 13.55 ± 0.12; $Z = 6.198$, $P < 0.0001$] and 2004 (Gainesville 14.95 ± 0.05, Ocala 13.81 ± 0.16; $Z = 6.785$, $P < 0.0001$). The differences in distributions were evident in the average ratio of polygyne (≤ 12 mg): monogyne (≥ 14 mg) NMJs with 0.21:0.53 at the Ocala collection site and 0.03:0.80 at the Gainesville collection site for the two years. This is consistent with the area surrounding our NMJ collection sites in Gainesville having a higher proportion of monogyne colonies, since monogyne colonies produce more and heavier female alates. Table 1 shows the distribution of NMJs within the designated weight classes and the percentage of NMJs collected in each weight class for each year and collection site. The number of NMJs collected at the Ocala site was very different for the two collection years; however, the percent NMJs distributed in each weight class was similar (Table 1). The number of NMJs collected in Gainesville was similar, as were the percentages NMJs in each weight class (Table 1).

3.2. Determination of social form

A random sample of 96 NMJs weighing ≥ 14 mg collected from the Gainesville site were all found to have the BB genotype for the *Gp-9* locus, while a random sample of 48 NMJs weighing ≤ 12 mg collected from the Ocala site included 41 Bb, 6 BB and 1 bb individuals for the *Gp-9* locus. At the NMJ higher weight category the lack of the b

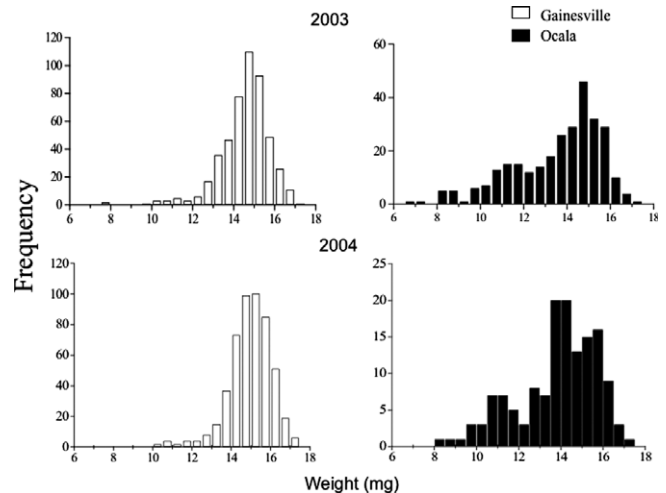


Fig. 1. The frequency of newly mated queens for different weight classes, collected from a primarily monogyne population site in Gainesville, and a predominately polygyne area in Ocala. See Table 1 for total newly mated queens collected per site and per year.

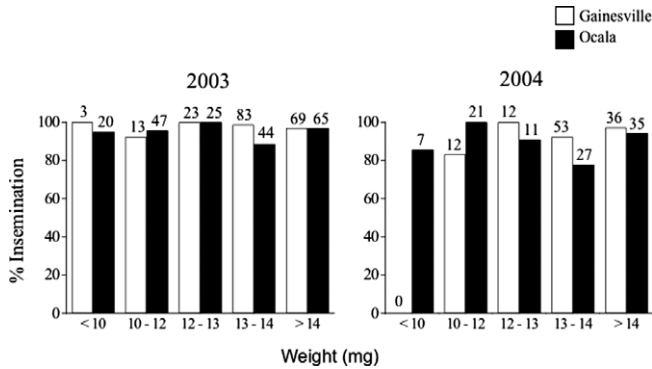


Fig. 2. The percentages of insemination of newly mated queens collected in Gainesville and Ocala from mating flights in 2003 and 2004. The n value above each bar indicates the number of newly mated queens examined for insemination within each weight class (see also Table 1).

allele supports our assignment of monogyne form to NMQs within this heavier weight category. For the lower weight NMQ category over 85% of the time the NMQ was correctly assigned to the polygyne Bb category.

3.3. Insemination rates at two collection sites

For all collections, insemination levels were high, ranging from 90.1% to 97.91%. The proportion of inseminated NMQs was not significantly different between the Gainesville and Ocala field sites in 2003 (Fig. 2; $\chi^2 = 0.317$, $df = 390$, $P = 0.075$) or 2004 ($\chi^2 = 1.002$, $df = 212$, $P = 0.317$, respectively). There was no significant difference in insemination rates between monogyne NMQs ≥ 14 mg or polygyne NMQs ≤ 12 mg at either site in either year ($\chi^2 = 0.370$, $2,492$, $df = 83,46$, $P = 0.543$, 0.115 , Gainesville 2003, 2004, respectively; $\chi^2 = 0.649$, 0.161 , $df = 130,61$, $P = 0.420$, 0.688 , Ocala 2003, 2004, respectively). Since we use monogyne NMQs collected in Gainesville and polygyne NMQs collected in Ocala for behavioral experiments, we were interested in knowing if there was a difference in the number of NMQs inseminated between this specific social form—collection site combination. There was no difference in 2003 ($\chi^2 = 0.773$, $df = 134$, $P = 0.379$) or 2004 ($\chi^2 = 0.033$, $P = 0.857$); therefore, insemination rate is not affected by social form at these collection sites.

3.4. Infection rates at two field sites

The proportion of NMQs infected with *T. solenopsae* was significantly greater at the Ocala field site in both 2003 (Fig. 3; $\chi^2 = 19.889$, $df = 390$, $P < 0.0001$) and 2004 (Fig. 3; $\chi^2 = 8.384$, $df = 212$, $P < 0.0038$). Significantly more polygyne NMQs (≤ 12 mg) were infected than monogyne NMQs (≥ 14 mg) ($\chi^2 \geq 5.775$, $df \geq 46$, P 's ≤ 0.0163) for 2003 and 2004 Gainesville data; however, no comparison between social forms was possible within the 2003 collection from Gainesville because there were no infections in the high and the low NMQ weight categories (Fig. 3).

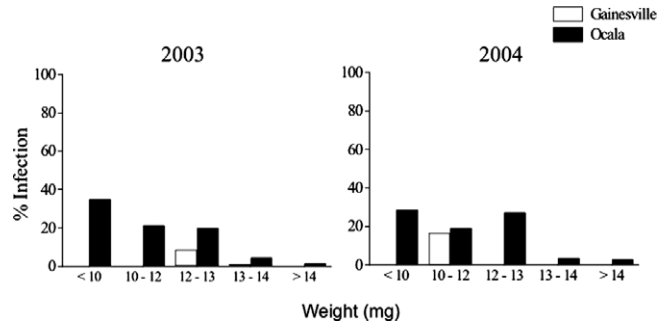


Fig. 3. The percentages of infection with *Thelohaniasolenopsae* of newly mated queens collected in Gainesville and Ocala, FL from mating flights in 2003 and 2004. The number of newly mated queens examined for infection within each weight class are the same as indicated in Fig. 2 for insemination rate (see also Table 1).

4. Discussion

Polygyne-derived NMQs are putatively incapable of initiating colony formation on their own, due primarily to their lower energy reserves (Keller and Passera, 1989). Although DeHeer, 2002 showed that 65% of single polygyne NMQs were able to produce at least one worker, their survival and development into functional colonies was not followed and is unknown. As pointed out in section 1, the number of nanitic workers produced by NMQs is critical to colony development. In association with other experiments we have been unable to produce viable colonies from clausurally founding low weight, *Gp-9^{Bb}*, NMQs (R.V.M. and C.A.P., unpublished data).

The vital fat reserves of female alates are negatively affected when they are infected with *T. solenopsae*. In previous studies the microsporidium *T. solenopsae* was found to infect 23% (86/379) of *S. invicta* colonies sampled in north central Florida (Williams et al., 1998, social form of sampled colonies not clearly defined, but all infected colonies stated to be polygyne). Until recently, *T. solenopsae* has only been detected in polygyne populations of *S. invicta*, although monogyne colonies can be infected through adoption of infected brood in the laboratory, resulting in reduced brood production and premature death of queens (Williams et al., 1999). Fuxa et al. (2005a) successfully infected monogyne field colonies and further identified 12 naturally infected monogyne *S. invicta* colonies in a 0.2-ha plot near Baton Rouge, Louisiana (Fuxa et al., 2005b). In both field situations the pathogen did not increase its prevalence and ultimately disappeared (Fuxa et al., 2005a,b). This microsporidium is capable of infecting female and male sexuals. Mature spores of *T. solenopsae* are found in pupae, adult workers, and queen ovaries, and vegetative stages are found in larvae, pupae, and queen ovaries (Knell et al., 1999; Sokolova and Fuxa, 2001; Shapiro et al., 2003) as well as in eggs, indicating that *T. solenopsae* is transmitted transovarially (Briano et al., 1996). Varying prevalence rates of alate infection have been reported, e.g. male (20.5%) and female (<25%; Cook et al., 2003); male (93.3%) and

female (75.2%; Oi and Williams, 2003). It appears that *T. solenopsae* infection negatively affected the weight of alates collected post-mating flight; however, the social form of the alates was not determined definitively (Cook et al., 2003). The rate of insemination was not affected by *T. solenopsae* infection, suggesting that infection status does not impact the ability of alates to successfully participate in mating flights (Cook et al., 2003).

The low weight polygyne-derived NMQs were collected from population-wide mating flights at both study locations, thus their low weight did not prevent them from flying. Similarly, >90% of these NMQs were able to find a mate and were inseminated. A possible explanation for their lack of colony-founding success is that the polygyne NMQs were infected with *T. solenopsae*. However, the polygyne NMQs collected in Ocala, defined here as weighing ≤ 12 mg had infection levels of about 23% (Fig. 3). Therefore, infection by *T. solenopsae* cannot be completely responsible for the inability of polygyne NMQs to claustrally establish colonies. It is most likely that the reduced fat reserves associated with the polygyne social form, which in turn result in low numbers of first workers, are primarily responsible for their lack of success in independent colony foundation, although other factors may also be involved.

We found only two NMQs (out of 205) in the ≥ 14 mg weight class, presumably *Gp-9^{BB}*, that were infected with *T. solenopsae*. Previous investigations only identified *T. solenopsae* in polygyne populations in the field and not in monogyne (Oi et al., 2004). However, Fuxa et al. (2005b) recently reported transient *T. solenopsae* infections in monogyne colonies in a mixed social form population, which could provide a source for *T. solenopsae* infected monogyne-derived NMQs. Another possibility is that our infected, ≥ 14 mg NMQs represent heavy, polygyne-derived *Gp-9^{BB}* NMQs. Oi et al. (2004) did find infected monogyne genotype, *Gp-9^{BB}*, female alates from polygyne colonies. Interestingly, no polygyne-derived *Gp-9^{BB}* queens have been found to head monogyne colonies (DeHeer et al., 1999).

Polygyne colonies show reduced production of male and female sexuals (Vargo and Fletcher, 1987), which is additionally complicated by production of sterile diploid males (Ross and Fletcher, 1985, 1986; Ross, 1992; Krieger et al., 1999). Consequently, virtually all polygyne queens mate with monogyne-derived males (Ross and Shoemaker, 1993), requiring that the polygyne social form exists in close proximity to the monogyne form. The low insemination rates found by Cook et al. (2003) for infected and non-infected (65% and 69%, respectively) NMQs in a geographic area where monogyne colonies are rare supports the importance of monogyne source males for successful polygyne-derived female alate mating, and supports our finding that *T. solenopsae* infection does not affect mating flight participation and mating. Ross and Shoemaker (1993) showed that polygyne queens were exclusively mated with monogyne-derived males. Our polygyne collection site

is a mosaic of monogyne and polygyne colonies (Fritz and Vander Meer, 2003) and our high insemination rates at the polygyne and monogyne dominant sites (95%) reflect an abundance of viable male alates.

Variability in *T. solenopsae* prevalence rates emphasizes the need to determine *T. solenopsae* prevalence at experimental field sites when infection status may have a contributing effect on unrelated experimental results. Moreover, the sampling design must be relevant to the scope of the study, as prevalence rates vary depending on what is being sampled. In Gainesville, Florida, there was an average prevalence level of 81.8% (45 of 55) of polygyne colonies sampled (Oi et al., 2004), an average of approximately 46% of inseminated queens collected from nine polygyne colonies were infected (Oi and Williams, 2002), and 7.13% (47/659) of colonies reared from NMQs collected over a four year period were infected by *T. solenopsae* (Oi and Williams, 2003). Cook (2002) found significant spatial and temporal variation in the prevalence of infected polygyne colonies in Fort Walters, Parker County, Texas, with infection levels of 0%, 21%, 30%, 33%, 67%, and 68% over a two year period. Female alate infection prevalence rates in Huntsville, Walker County, Texas, were less than 25% in four collections over a 13 month period (Cook et al., 2003). It is now difficult to find polygyne populations that are not infected with *T. solenopsae*. With the recent identification of infected monogyne colonies by Fuxa et al. (2005b), infection levels should be considered regardless of the social form of the experimental colonies. In addition, phorid fly parasites are now established and spreading in most of the fire ant infested areas of the United States. Recently, Valles et al. (2004) reported the discovery of a picorna-like virus, tentatively named *S. invicta* virus (SINV-1) and their surveys of natural populations found a 25% prevalence rate. Unlike the microsporidian *T. solenopsae*, this virus is prevalent in both social forms. These sustainable parasites and pathogens and others that may be introduced in the future must be considered when research involves the use of natural populations, whether directly in field-based experiments, or indirectly when natural colonies or components of such are used in laboratory experiments.

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