Short independent lives and selection for maximal sperm survival make investment in immune defences unprofitable for leaf-cutting ant males

Running title: Fitness trade-offs in ant males

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Abstract

The short-lived males of ants and other highly eusocial Hymenoptera are essentially ejaculates with compound eyes, brains and wings to vector sperm to its destination. Males compete for life-time ejaculate storage by females to produce the equivalent of somatic cells (sterile workers) and new seed-propagules (gynes; males are haploid and have no father) after the colony has become sexually mature. Hymenopteran queens never re-mate later in life, which makes partner commitment between queen and male-ejaculate analogous to a sperm and egg committing when forming a zygote that subsequently sequesters a germ line and produces somatic tissues. This semelparous commitment remains unchanged when queens store ejaculates from multiple males and colonies become chimeras of patriline. The soma of eusocial hymenopteran males may thus not be under selection for more than minimal independent life, but eusocial male ejaculates are unusually long-lived and sperm cells may not be used until years after storage. Somatic repair and immune defence in males should thus be minimal, particularly in response to challenges late in adult life. We tested this idea using males of *Atta* and *Acromyrmex* leaf-cutting ants and show that lethal infections with the fungal pathogen *Metarhizium brunneum* affect male sperm quality, but fail to induce an encapsulation immune response. This result is consistent with expectation because fungal infections are highly unlikely to ever reach immature ant males while they are nursed by their sister workers, and because males will die natural deaths after leaving their colonies to mate before new infections can kill them.

Key words: *Metarhizium brunneum*, immunity, encapsulation response, *Atta colombica*, *Acromyrmex echinatior*
The most advanced eusocial insects have colonies with (super)organismal properties (Hölldobler and Wilson 2008; Queller and Strassmann 2009) where collective actions of sterile workers produce emergent properties that protect against fluctuations in temperature, humidity and disease pressure (Wilson 1971; Cremer et al. 2007; Seeley 2010; Konrad et al. 2012). In ants and termites, colonies can be extremely long-lived (Keller and Genoud 1997), because mortality of established queens is very low, but early in life the dispersing virgin queens (gynes) generally suffer huge mortality once they leave the protective boundaries of their colony to mate and disperse (Hölldobler and Wilson 1990). Particularly the reproductive life-history of ant queens and their colonies has interesting analogies with the initiation and development of metazoan bodies. Queens store ejaculates for life just like an animal egg incorporates a sperm cell upon fertilization, after which an incipient ant colony produces sterile workers reminiscent of somatic tissues (Boomsma 2013).

Leaf-cutting ants have some of the longest lived colonies with queen life-spans up to 20 years (Weber 1972; Den Boer et al. 2009), but males die on the day they leave the colony in which they hatched, having lived as adults for just a few weeks under constant protection by their worker sisters who provide sophisticated social immunity against external parasites and pathogens (Hughes et al. 2002; Cremer and Sixt 2009).

Spermatogenesis ceases once a male ecloses from its pupa (Hölldobler and Bartz 1985), but stored sperm remain viable throughout the life-span of a queen. This implies that the sperm of ant males has been selected to be highly viable (Boomsma et al. 2005a; Den Boer et al. 2009), but that somatic tissues of males may not be adapted to any other
function of independent life than finding a mate and delivering high quality semen with maximal efficiency. This is analogous to metazoan sperm cells that are produced and stored in highly protective testicular tissues, but have very short independent lives after ejaculation, although each male gamete can potentially fertilize an egg to produce a zygote that may develop into a long-lived body before it reproduces. In contrast, the long-lived queens of leaf-cutting ants show a clear trade-off between the maintenance of stored sperm and immune defence during solitary colony founding (Baer et al. 2006). This extreme contrast offers interesting possibilities to test how trade-offs between reproduction and maintenance differ between eusocial queens and males.

As pathogens are ubiquitous in almost all natural environments, immune defences represent one of the most universal investments in somatic maintenance. Immune responses are known to be costly, both the expression of innate immunity and the induction of immune responses in reaction to specific pathogen challenges (Sheldon and Verhulst 1996; Moret and Schmid-Hempel 2000; Siva-Jothy and Thompson 2002; Ardia et al. 2012). Many studies have documented that immune activation is traded off against growth and reproduction (Rolff and Siva-Jothy 2002; Schmid-Hempel 2005; van der Most et al. 2011; Simmons 2012; McNamara et al. 2013), so it is no surprise that queens of Atta leaf-cutting ants activate their immune defences when they initiate their solitary burrows immediately after mating (Baer et al. 2006). Until they will have produced their first workers to initiate foraging some months later, founding queens are severely resource-constrained, consistent with investment in immune defence being negatively correlated with the amount of stored sperm that needs to be maintained (Baer et al. 2006).
In contrast to queens, males of eusocial bumblebees and ants are known to have reduced immune responses compared to workers (Gerloff et al. 2003; Vainio et al. 2004; Baer et al. 2005), consistent with reduced investment in their maintenance outside the protective colony in which they hatched. Although there may not be a single optimal design for ant males within a species (Stürup et al. 2011), it is interesting to explore the extent to which male ant phenotypes have become generally dependent on the special eusocial context in which they develop and hatch before they leave their colonies to mate. As natural selection is not expected to establish or maintain traits to cope with conditions that never occur naturally, it would seem logical that immune defences reflect the average sterility/infectivity of male rearing conditions. This would imply that infections with generalist pathogens that males are not exposed to when cared for by workers might fail to induce immune defences when males only have some hours more to live after leaving their colonies, particularly when the cost of such responses would jeopardize sperm viability. Trade-offs between sperm viability and immunity have been documented for fruit flies, where pathogen inoculation decreases sperm viability in both sexes (Radhakrishnan and Fedorka 2012), and crickets where juvenile bacterial exposure translated into reduced sperm viability in adult males (Simmons 2012). These responses seem logical as male reproductive success will depend on the number of females they mate with throughout their adult lives, whereas ant males are semelparous and will have all their mating activities on a single day. To experimentally test whether eusocial insect males do indeed have unresponsive immune systems when they have become sexually mature, we inoculated males of two species of leaf-cutting ants, *Atta colombica* and *Acromyrmex echinatior*, with the naturally occurring entomopathogenic fungus *Metarhizium brunneum*. 
our test species *At. colombica* and *Ac. echinatior* co-occur around Gamboa, Panama, and have similar life histories, maximal colony longevities of 10-20 years and multiple mating of queens (Weber 1972; Sumner *et al.* 2004; Den Boer *et al.* 2009; Holman *et al.* 2011). However, the two species differ approximately two orders of magnitude in mature colony size and their males are about an order of magnitude different in body mass (Villesen *et al.* 2002; Dijkstra *et al.* 2005; Stürup *et al.* 2011). Our experimental setup allowed us to test whether: (1) males are vulnerable to infections with fungal pathogens, (2) infections compromise potential male reproductive fitness, and (3) immunocompetence co-varies with sperm viability. Infection with *Metarhizium* and mild stressors such as light wounding and elevated temperatures are known to negatively affect sperm viability in honeybee drones (Stürup *et al.* 2013). However, in the present study we measured the encapsulation response shortly after inoculation to explicitly test whether induced immunity affects sperm viability if it occurs at all.

### Methods

We excavated colonies of *At. colombica* and *Ac. echinatior* leaf-cutting ants in Gamboa Panama, Republic of Panama. We collected entire *Acromyrmex* colonies including the founding queen and transported them to Copenhagen, Denmark where they were housed in climate rooms at ca. 25°C and 70% RH. Captive *Acromyrmex* colonies can be maintained in the lab for more than 10 years and reach sizes where they often produce substantial numbers of male and female (gyne) sexuals once or twice a year (log-book data, CSE Copenhagen). However, mother queens of fully grown *At. colombica* colonies are virtually impossible to collect in the field, and incipient queen right colonies never reach sizes in the laboratory that are sufficient to produce sexuals. We
thus conducted our experiments with *Ac. echinatior* in the lab in Copenhagen and our experiments with *At. colombica* in Gamboa, but otherwise in the same manner for both species, using three colonies of *Ac. echinatior* and six colonies of *At. colombica* (three colonies each in two consecutive years). *Acromyrmex* colonies with males and workers were maintained in fluon coated plastic boxes on a standard diet of bramble leaves and rice (Baer *et al.* 2005; Dijkstra *et al.* 2005). Males of *Atta* were kept in plastic trays at ambient Panamanian temperature and humidity together with workers and fungus garden material until being processed within six days after excavation.

**Nylon implants**

To quantify the immunocompetence of males we measured encapsulation responses against implanted nylon filaments (Baer *et al.* 2005; Baer *et al.* 2006). These responses are initiated when haemocytes recognize and attach themselves to foreign objects while forming hard melanin capsules around them, responses that have been shown to correlate with insect resistance against pathogens (Rantala and Roff 2007).

Encapsulation responses are initiated 4-6 hours after non-self particles (here a nylon implant) enter tissues and continue over a period of 48 hours (Eslin and Prevost 2000). We followed the protocol previously used by Baer *et al.* (2005), anesthetizing males with CO₂ and fixing them in a plastic holder of modified equipment for the artificial insemination of bumblebees (Baer and Schmid-Hempel 2000). Once anesthetized, male sternites were then gently separated using two blunt hooks, after which the intersegmental membrane was punctured with a sterile syringe needle and a nylon filament (0.13 x 1 mm for *Atta* and 0.13 x 0.5 mm for *Acromyrmex*) inserted between the 2\textsuperscript{nd} and the 3\textsuperscript{rd} sternite.
Males were allowed to recover from the nylon implant procedure and then allocated to treatment groups of either *M. brunneum* (formerly *M. anisopliae*; Bischoff et al. 2009) inoculation (strain KVL 04-57) or talcum powder treatment (controls) and placed in Petri dishes with a moist piece of cotton wool and access to *ad libitum* water. We used *M. brunneum* as this generalist pathogen is known to predictably kill leaf-cutting ants in high-dose experiments (Yek et al. 2013), and is present in the soil where we excavated the colonies (Hughes et al. 2004). Using a fine paintbrush we transferred dry fungal conidia (asexual spores) taken from 0.5 cm² of pure fungal cultures grown on potato dextrose agar (PDA) medium to the thorax of males, inoculating them with $7 \times 10^6$ spores per six *Acromyrmex* males and $7 \times 10^6$ spores per single *Atta* male. Spores were applied to the dorsal side of the male’s torso and control group males received an equivalent dose of talcum powder in the same location. Conidia of *M. brunneum* initially adhere to the cuticle of the host insect after which they germinate and penetrate into the cuticle and haemocoeal within 24-48 hours (Boucias and Pendland 1998). We checked spore viability before the experiments by plating 100 μl of a $10^5$ spores/ml suspension from the same culture onto a PDA plate and counting the proportion of germinating conidia after 24 hours.

After inoculations males were placed in Petri dishes (60 x 15 mm) together with brothers of the same treatment group (two for *At. colombica* and six for *Ac. echinatior*), but without workers because their allogrooming would reduce infection efficiency (Schmid-Hempel 2005; Walker and Hughes 2009; Konrad et al. 2012). Ant males do not generally participate in allogrooming (Wilson and Fagen 1974) and our own
observations confirmed this for *Ac. echinatior* and *At. colombica*. Males were supplied with *ad libitum* access to water and were left undisturbed in Petri dishes with small holes in the lids securing ventilation until they were processed. Half of the males were dissected after one day and the other half after two days. Males were generally confirmed to be sexually mature during dissections, because their accessory testes were fully developed and their testes degenerated (Hölldobler and Bartz 1985; Baer and Boomsma 2006). However, we discarded some males from further analyses when this turned out not to be fully the case or when some of their sperm had already been transferred to the accessory glands as normally happens when they are about to ejaculate (Baer and Boomsma 2004). Total sample sizes ended up being 20 and 36 males of *Ac. echinatior* one and two days after treatment, respectively, and 89 and 79 males of *At. colombica* one and two days after treatment, respectively.

**Dissections**

We anesthetized the males with CO₂, weighed them down to the nearest mg and dissected out their entire reproductive organs under a stereomicroscope. The accessory glands were separated from the accessory testes and both glands were transferred to an eppendorf tube and their content thoroughly mixed in 500μl Hayes saline solution (9 g NaCl, 0.2 g CaCl₂, 0.2 g KCl, and 0.1 g NaHCO₃ in 1,000 ml H₂O (Den Boer *et al.* 2008)). We then collected 1μl sperm from the accessory testes in a pipette, transferred this to the eppendorf and gently diluted the sperm in the solution. Hayes has previously been used as a semen diluent for insect sperm (Den Boer *et al.* 2008; Den Boer *et al.* 2010), and although the handling of the ejaculates and the exposure to the solution likely decreases sperm viability from what it is *in vivo* (Holman 2009) all ejaculates...
received the same treatment, allowing us to compare relative sperm viabilities (survival %) between treatment groups.

Sperm viability assessment and encapsulation response measurements

We measured the proportion of live sperm using a Live/DeadTM sperm viability kit (L-7011, Molecular Probes) that consists of a membrane-permeant nucleic acid that stains DNA green (SYBR 14) and a non-permeant stain (propidium iodide) that stains dead sperm cells red (Holman 2009). We left the semen samples to incubate for an hour at 25°C after which we gently mixed the content and pipetted 2 x 5μl of the sperm dilution onto a microscope slide, added 5μl of SYBR 14 and incubated the sample in the dark for 10 minutes. We then added 2 μl propidium iodide, applied a cover slide to the droplet and incubated the sample for another 7 minutes in the dark. The number of live and dead sperm cells was counted using a fluorescence microscope (Olympus CX41, EXFO X-Cite 120, 400 x magnification) for a minimum of 300 sperm per sample. For statistical analyses we averaged the counts for the two accessory testes samples for each male.

During the dissections we retrieved the nylon filament from the males, except for seven of the 168 At. colombica males and four of the 56 Ac. echinatior males where the nylon piece could not be found again. We measured the encapsulation response by mounting each retrieved nylon filament onto a microscope slide next to a filament which had not been implanted, and photographed each filament using a Nikon DS-Fi1 camera connected to a Nikon SMZ 800 microscope (Leica MZ12 dissecting microscope connected to a Leica DFC 420 camera for Acromyrmex). The grey scale of implanted and control filaments was measured using Image J software (freely available at
Encapsulation response was then calculated as the grey value difference between implanted and control filament.

Statistics

We analyzed data using JMP software version 9.0.2 for Windows. Sperm viability counts were logit transformed to normalize the distribution. For the analysis we used ANCOVA, including *Metarhizium* infection, ant species and their interaction as main effects (with species defined as a random effect). Year (nested within “Species”) and Colony (nested within “Year and Species”) were included as random factors. The measured variables “fresh weight”, “encapsulation response” and “days after inoculation” were included as covariates, together with their interactions with the main effects “*Metarhizium* infection” and “Species”. Non-significant covariates were then removed from the model by backward elimination unless they had a significant interaction with main effects. Since not every covariate was recorded for every male, the sample sizes and error degrees of freedom changed slightly between models (see Table 1A and B, full and reduced model respectively). To test the effect of the fungal infections on male survival we ran parallel inoculation experiments measuring time to death for *Metarhizium* inoculated versus talcum control treated males, using males from all colonies. The effects of species, treatment and colony (nested within species) on survival were analyzed using a proportional hazards regression model. Sperm viability counts and encapsulation responses were measured blind to treatment.

Results

Germination success of the *M. brunneum* conidia (asexual spores) was 99.3 ± 0.08 % similar to Yek *et al.* (2013). Inoculations with this fungal pathogen significantly
reduced male survival for both *At. colombica* and *Ac. echinatior* compared to the talcum treated control males (Effect likelihood ratio (LR) $\chi^2 = 109.06$, d.f. = 1, $p < 0.0001$). The average rate of survival differed between species (LR $\chi^2 = 4.42$, d.f. = 1, $p = 0.036$), but the difference between *At. colombica* across years was not significant (year nested within species, LR $\chi^2 = 0.37$, d.f. = 1, $p = 0.54$). Reduced survival after *Metarhizium* inoculation was consistent across all colonies (colony nested within year and species x *Metarhizium* inoculation, LR $\chi^2 = 8.41$, d.f. = 6, $p = 0.21$).

Males of both *At. colombica* and *Ac. echinatior* inoculated with *Metarhizium* had significantly reduced sperm viability values compared to the respective control groups ($p < 0.0001$, Table 1A, Figure 2) and this outcome was consistent across species as the species x treatment interaction was non-significant ($p = 0.85$, Table 1A). We found no significant effect of time-span between treatment and sperm viability measurements, but sperm viability in infected *At. colombica* males was significantly reduced already after 1 day whereas this took 2 days in *Ac. echinatior* males (species x days after inoculation, $p = 0.0037$, Table 1A). This difference might be related to the background mortality in *At. colombica* males being higher than in *Ac. echinatior* males (Figure 1).

Encapsulation responses differed significantly between colonies (nested within year and species; $p < 0.0001$, Table 1B, Figure 3), but not between infected and non-infected males of both species ($p = 0.92$, Table 1B). Encapsulation response was not a significant predictor of sperm viability in either species ($p = 0.68$, Table 1A) and was also not significantly different between *Metarhizium* treated individuals and controls (Figure 3, Table 1B).
We found that males of both leaf-cutting ant species died 2-4 days after inoculation with *Metarhizium* spores, in contrast to *Ac. echinatior* workers which take 4-6 days to die after higher inoculation doses (Yek et al. 2013). The infected males did not show any signs of an up-regulated immune response in spite of their sperm quality being compromised by the infection. This result matched our expectations, because both sperm maintenance and immune responses are costly, so a balanced investment only pays off when somatic survival is also important for reproductive success, as for example in founding queens of *At. colombica* (Baer et al. 2006). These findings also confirm earlier results for *Ac. echinatior* showing that male encapsulation response is always lower than that of nestmate-workers (Baer et al. 2005). Thus, somatic survival does not appear to be a target for selection when males predictably die from disease after 2-4 days, well beyond their residual life-span of less than a day when leaving the nest to mate.

Our results imply that ant males are vulnerable and generally unfit for independent life functions other than delivering high quality ejaculates, a notion that was confirmed by the need to do the inoculations and nylon implantations in a single treatment after pilot experiments had shown that double-handling the males (e.g. inserting nylon filaments 24 hours after inoculations) invariably killed all males before a response could be measured. This precluded repeating the experiment inserting the nylon implants one day after inoculation which would have allowed us to examine the melanisation response of infected males in a fully controlled manner, as melanisation may have already partly proceeded before infection in our experiments. However, already after one day we measured negative treatment effects on sperm, which can only be explained by the
immune challenge starting within hours after inoculation. As saturation of encapsulation responses in *Drosophila* did not appear until 48 h after inoculation (Eslin and Prevost 2000), we therefore assumed that both our first and second day measurements reflected relevant immune defences.

Although we could not find evidence for induced immune responses, there was phenotypic variation for innate melanisation response late in male adult life across colonies, suggesting that rearing conditions vary and that some genetic variation for the expression of innate immune response was present. Also this type of variation has been shown previously in a study comparing the magnitude of encapsulation differences between males and workers of *Ac. echinatior* (Baer et al. 2005).

Genetic disadvantages of haploidy have been suggested to make hymenopteran males more prone to infections, so they should avoid contact with pathogens (O'Donnell and Beshers 2004) and thus remain unfit for foraging and nursing tasks. Yet, Gerloff et al. (2003) found no immune response differences between haploid and diploid males and a comparative study across social bees by Ruiz-Gonzalez and Brown (2006) also questioned the haploidy disadvantage idea. Our results suggest instead that the extent to which eusocial males have independent lives outside their colony of origin is crucial, which implies the prediction that long-lived patrolling males of bumblebees (Alcock et al. 1978; Thornhill and Alcock 1983; Schmid-Hempel 1998) should upregulate their immune defences if they would contract infections after leaving their colony. This ability would then likely be linked to a higher need to express immune defences also before leaving the nest, as annual bumblebee colonies live with their diseases, whereas perennial ant colonies are able to almost entirely exclude them through advanced social
immunity mechanisms (Boomsma et al. 2005b; Cremer and Sixt 2009; Ugelvig et al. 2010). This is supported by bumblebee males raised in parasite-challenged colonies responding by up-regulating their constitutive immune system (Moret and Schmid-Hempel 2001) although their immune response remains generally lower than that of workers (Gerloff et al. 2003; Baer and Schmid-Hempel 2006).

In contrast to bumblebees (Wilson 1975), leaf-cutting ants have elaborate allogrooming behaviours involving the use of antiseptic metapleural gland secretions that ensure almost disease free nest interiors (Hughes et al. 2002; Poulsen et al. 2002; Boomsma et al. 2005b; Yek and Mueller 2011). This likely removes most selection for any inducible immune responses, consistent with seeing isolated males invariably succumb to infection in our experiments after removing their worker-mediated social immune defences. A previous study (Stürup et al. 2013) showed that sperm quality of honeybee drones is also very sensitive to mild abiotic and immune challenges, suggesting that similar adaptations have convergently evolved, with males being unfit for independent (unprotected) life as inevitable consequence. It is interesting to note that large-scale male aggregation syndromes during mating flights (sensu Hölldobler and Bartz 1985) appear to have evolved only in perennial eusocial insects (all ants, all termites, stingless bees, honeybees). This suggests that there may be a connection between advanced social immunity and largely germ-free rearing of males on one hand, and the very short independent lives of males after leaving their colonies to disperse and mate on the other.

Some eusocial insect males are long-lived by default or evolved longer life-spans secondarily. Eusociality in termites is based on monogamous commitment of males to queens during colony founding (Boomsma 2013) but with males being equally long-
lived as queens. Termite males thus continue to produce sperm similar to queens continuing to produce eggs, so immunocompetence and investment in somatic repair would not be expected to differ between the sexes. This has been partly confirmed by Calleri *et al.* (2007) who found that male encapsulation responses in the dampwood termite *Zootermopsis angusticollis* were in fact higher than those of queens.

A single ant genus, *Cardiocondyla*, has secondarily evolved longer-lived males (Heinze and Hölldobler 1993), i.e. ergatoid male morphs that continue to produce sperm throughout life to mate with virgin queens in the same nest. These males live reproductive lives of up to one year before being superseded by younger nestmates (Yamauchi *et al.* 2006; Suefuji *et al.* 2008), so have intermediate life-spans between the males of other ants and those of termites (Boomsma *et al.* 2005a). Yet, similar to established termite reproductives, these ergatoid males never operate without having nursing workers nearby, so their operational immune defences will likely depend on the efficiency of the social immunity that workers can provide (Cremer and Sixt 2009; Rosengaus *et al.* 2011). It would thus be interesting to find out whether they can upregulate immune defence when challenged.

The sexes may have different life spans in solitary insects (Fox *et al.* 2003), but never to the same extent as in the perennial eusocial Hymenoptera such as ants, honeybees and stingless bees. Our results on leaf-cutting ants and comparisons with other eusocial insects suggest that there is a fundamental connection between size and longevity of eusocial colonies, prophylactic disease management, and the longevity and robustness of males, the sex that is increasingly reduced to (and specialized on) semelparously transferring sperm as eusociality becomes more advanced.
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References


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Boomsma JJ (2013) Beyond promiscuity: mate-choice commitments in social breeding. Philos T R Soc B 368:


Figure 1

Average survival curves of males from the six *At. colombica* and three *Ac. echinatior* colonies, after inoculation with *M. brunneum* spores (dashed lines) or talcum powder controls (solid lines). Control mortality in the *Atta* experiments was substantial, possibly because *Atta* males were more stressed as they had to be excavated in the field whereas *Acromyrmex* males were collected from established lab colonies without significant disturbance. However, this should not have affected our results, as all experiments were completed by day 2 when control survival was still close to 100%.

Figure 2

Proportions of male sperm alive in talcum powder controls and *Metarhizium* inoculated males of *Ac. echinatior* (dashed lines) and *At. colombica* (solid lines) in 2012 (closed symbols) and 2013 (open symbols). Sperm viability was measured 1 and 2 days after inoculations respectively. Means are based on un-transformed sperm viability counts and thin dotted lines connect the same colonies between the left and right panels.

Figure 3

Mean grey scale values measuring encapsulation responses in talcum powder controls and *Metarhizium* inoculated males of *Ac. echinatior* (dashed lines) and *At. colombica* (solid lines) in 2012 (closed symbols) and 2013 (open symbols). Nylon filaments were retrieved 1 and 2 days after inoculations, respectively, and thin dotted lines connect the same colonies between the left and right panels.
Result of ANCOVAs to explain variation in sperm survival (logit transformed proportions live sperm) (A) and encapsulation responses (B) in males of *Atta colombica* and *Acromyrmex echinatior*. *Metarhizium* infection, species and their interaction were included as main effects, with species, year and colony included as random factors, with the latter two nested within species and years (terms in parentheses). Encapsulation response (only Table A), days after inoculation and fresh weight of males were included as covariates, together with their interactions with the main effects. Model simplification was carried out by the backward elimination of non-significant covariates and their interactions. Statistics in the upper parts of the tables refer to reduced models after the variables in the lower parts were eliminated because they were not significant. Statistics and p-values are reported for “Full model” terms immediately prior to their removal. As not all covariates were measured for all males, the sample sizes and error degrees of freedom vary depending on the included covariates.

**Table 1**

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<td>Reduced model</td>
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<td>Species</td>
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<td>Colony (Year &amp; Species)</td>
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<td>Days after inoculation</td>
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Proportion males alive +/- SE

Figure 1

**Atta colombica**

- **Days after treatment**: 0, 1, 2, 3, 4
- **Proportion males alive**: 1.00, 0.74, 0.47, 0.20, 0.01
- **N**: 312

**Acromyrmex echinatior**

- **Days after treatment**: 1, 2, 3, 4
- **Proportion males alive**: 1.00, 0.74, 0.47, 0.20
- **N**: 150

Legend:
- Solid line: Live males
- Dashed line: Dead males
- Error bars: Standard error
Figure 2

Proportion live sperm +/- SE

1 day after treatment

2 days after treatment

Control

Metarhizium

Control

Metarhizium
Figure 3

1 day after treatment

2 days after treatment

Mean grey scale value +/- SE