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

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7 4 Running title: Fitness trade-offs in ant males
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26 **Abstract**

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

49 **Key words:** *Metarhizium brunneum*, immunity, encapsulation response, *Atta*
50 *colombica*, *Acromyrmex echinator*

51 **Introduction**

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53 The most advanced eusocial insects have colonies with (super)organismal properties
54 (Hölldobler and Wilson 2008; Queller and Strassmann 2009) where collective actions of
55 sterile workers produce emergent properties that protect against fluctuations in
56 temperature, humidity and disease pressure (Wilson 1971; Cremer *et al.* 2007; Seeley
57 2010; Konrad *et al.* 2012). In ants and termites, colonies can be extremely long-lived
58 (Keller and Genoud 1997), because mortality of established queens is very low, but
59 early in life the dispersing virgin queens (gynes) generally suffer huge mortality once
60 they leave the protective boundaries of their colony to mate and disperse (Hölldobler
61 and Wilson 1990). Particularly the reproductive life-history of ant queens and their
62 colonies has interesting analogies with the initiation and development of metazoan
63 bodies. Queens store ejaculates for life just like an animal egg incorporates a sperm cell
64 upon fertilization, after which an incipient ant colony produces sterile workers
65 reminiscent of somatic tissues (Boomsma 2013).

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67 Leaf-cutting ants have some of the longest lived colonies with queen life-spans up to 20
68 years (Weber 1972; Den Boer *et al.* 2009), but males die on the day they leave the
69 colony in which they hatched, having lived as adults for just a few weeks under constant
70 protection by their worker sisters who provide sophisticated social immunity against
71 external parasites and pathogens (Hughes *et al.* 2002; Cremer and Sixt 2009).
72 Spermatogenesis ceases once a male ecloses from its pupa (Hölldobler and Bartz 1985),
73 but stored sperm remain viable throughout the life-span of a queen. This implies that the
74 sperm of ant males has been selected to be highly viable (Boomsma *et al.* 2005a; Den
75 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).

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101 In contrast to queens, males of eusocial bumblebees and ants are known to have reduced
102 immune responses compared to workers (Gerloff *et al.* 2003; Vainio *et al.* 2004; Baer *et*
103 *al.* 2005), consistent with reduced investment in their maintenance outside the
104 protective colony in which they hatched. Although there may not be a single optimal
105 design for ant males within a species (Stürup *et al.* 2011), it is interesting to explore the
106 extent to which male ant phenotypes have become generally dependent on the special
107 eusocial context in which they develop and hatch before they leave their colonies to
108 mate. As natural selection is not expected to establish or maintain traits to cope with
109 conditions that never occur naturally, it would seem logical that immune defences
110 reflect the average sterility/infectivity of male rearing conditions. This would imply that
111 infections with generalist pathogens that males are not exposed to when cared for by
112 workers might fail to induce immune defences when males only have some hours more
113 to live after leaving their colonies, particularly when the cost of such responses would
114 jeopardize sperm viability. Trade-offs between sperm viability and immunity have been
115 documented for fruit flies, where pathogen inoculation decreases sperm viability in both
116 sexes (Radhakrishnan and Fedorka 2012), and crickets where juvenile bacterial
117 exposure translated into reduced sperm viability in adult males (Simmons 2012). These
118 responses seem logical as male reproductive success will depend on the number of
119 females they mate with throughout their adult lives, whereas ant males are semelparous
120 and will have all their mating activities on a single day. To experimentally test whether
121 eusocial insect males do indeed have unresponsive immune systems when they have
122 become sexually mature, we inoculated males of two species of leaf-cutting ants, *Atta*
123 *colombica* and *Acromyrmex echinator*, with the naturally occurring entomopathogenic
124 fungus *Metarhizium brunneum*.

Our test species *At. colombica* and *Ac. echinator* 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. echinator* 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. echinator* 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. echinator* 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 2nd and the 3rd sternite.

176 *Inoculations*

177 Males were allowed to recover from the nylon implant procedure and then allocated to
178 treatment groups of either *M. brunneum* (formerly *M. anisopliae*; Bischoff *et al.* 2009)
179 inoculation (strain KVL 04-57) or talcum powder treatment (controls) and placed in
180 Petri dishes with a moist piece of cotton wool and access to *ad libitum* water. We used
181 *M. brunneum* as this generalist pathogen is known to predictably kill leaf-cutting ants in
182 high-dose experiments (Yek *et al.* 2013), and is present in the soil where we excavated
183 the colonies (Hughes *et al.* 2004). Using a fine paintbrush we transferred dry fungal
184 conidia (asexual spores) taken from 0.5 cm² of pure fungal cultures grown on potato
185 dextrose agar (PDA) medium to the thorax of males, inoculating them with 7 x 10⁶
186 spores per six *Acromyrmex* males and 7 x 10⁶ spores per single *Atta* male. Spores were
187 applied to the dorsal side of the male's torso and control group males received an
188 equivalent dose of talcum powder in the same location. Conidia of *M. brunneum*
189 initially adhere to the cuticle of the host insect after which they germinate and penetrate
190 into the cuticle and haemocoel within 24-48 hours (Boucias and Pendland 1998). We
191 checked spore viability before the experiments by plating 100 µl of a 10⁵ spores/ml
192 suspension from the same culture onto a PDA plate and counting the proportion of
193 germinating conidia after 24 hours.

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195 After inoculations males were placed in Petri dishes (60 x 15 mm) together with
196 brothers of the same treatment group (two for *At. colombica* and six for *Ac. echinator*),
197 but without workers because their allogrooming would reduce infection efficiency
198 (Schmid-Hempel 2005; Walker and Hughes 2009; Konrad *et al.* 2012). Ant males do
199 not generally participate in allogrooming (Wilson and Fagen 1974) and our own

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200 observations confirmed this for *Ac. echinator* and *At. colombica*. Males were supplied
201 with *ad libitum* access to water and were left undisturbed in Petri dishes with small
202 holes in the lids securing ventilation until they were processed. Half of the males were
203 dissected after one day and the other half after two days. Males were generally
204 confirmed to be sexually mature during dissections, because their accessory testes were
205 fully developed and their testes degenerated (Hölldobler and Bartz 1985; Baer and
206 Boomsma 2006). However, we discarded some males from further analyses when this
207 turned out not to be fully the case or when some of their sperm had already been
208 transferred to the accessory glands as normally happens when they are about to
209 ejaculate (Baer and Boomsma 2004). Total sample sizes ended up being 20 and 36
210 males of *Ac. echinator* one and two days after treatment, respectively, and 89 and 79
211 males of *At. colombica* one and two days after treatment, respectively.

212 213 *Dissections*

214 We anesthetized the males with CO₂, weighed them down to the nearest mg and
215 dissected out their entire reproductive organs under a stereomicroscope. The accessory
216 glands were separated from the accessory testes and both glands were transferred to an
217 eppendorf tube and their content thoroughly mixed in 500µl Hayes saline solution (9 g
218 NaCl, 0.2 g CaCl₂, 0.2 g KCl, and 0.1 g NaHCO₃ in 1,000 ml H₂O (Den Boer *et al.*
219 2008)). We then collected 1µl sperm from the accessory testes in a pipette, transferred
220 this to the eppendorf and gently diluted the sperm in the solution. Hayes has previously
221 been used as a semen diluent for insect sperm (Den Boer *et al.* 2008; Den Boer *et al.*
222 2010), and although the handling of the ejaculates and the exposure to the solution
223 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/Dead™ 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. echinator* 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

<http://rsbweb.nih.gov/ij/>). Encapsulation response was then calculated as the grey value difference between implanted and control filament.

251

252 *Statistics*

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

271 **Results**

272 Germination success of the *M. brunneum* conidia (asexual spores) was 99.3 ± 0.08 %
273 similar to Yek *et al.* (2013). Inoculations with this fungal pathogen significantly

reduced male survival for both *At. colombica* and *Ac. echinator* compared to the talcum
 treated control males (Effect likelihood ratio (LR) $\chi^2 = 109.06$, d.f. = 1, $p < 0.0001$,
 Figure 1). 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. echinator* 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. echinator* 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. echinator* 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).

Discussion

We found that males of both leaf-cutting ant species died 2-4 days after inoculation with *Metarhizium* spores, in contrast to *Ac. echinator* 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. echinator* 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. echinator* (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-

375 lived as queens. Termite males thus continue to produce sperm similar to queens
 376 continuing to produce eggs, so immunocompetence and investment in somatic repair
 377 would not be expected to differ between the sexes. This has been partly confirmed by
 378 Calleri *et al.* (2007) who found that male encapsulation responses in the dampwood
 379 termite *Zootermopsis angusticollis* were in fact higher than those of queens.
 380
 381 A single ant genus, *Cardiocondyla*, has secondarily evolved longer-lived males (Heinze
 382 and Hölldobler 1993), i.e. ergatoid male morphs that continue to produce sperm
 383 throughout life to mate with virgin queens in the same nest. These males live
 384 reproductive lives of up to one year before being superseded by younger nestmates
 385 (Yamauchi *et al.* 2006; Suefuji *et al.* 2008), so have intermediate life-spans between the
 386 males of other ants and those of termites (Boomsma *et al.* 2005a). Yet, similar to
 387 established termite reproductives, these ergatoid males never operate without having
 388 nursing workers nearby, so their operational immune defences will likely depend on the
 389 efficiency of the social immunity that workers can provide (Cremer and Sixt 2009;
 390 Rosengaus *et al.* 2011). It would thus be interesting to find out whether they can
 391 upregulate immune defence when challenged.
 392
 393 The sexes may have different life spans in solitary insects (Fox *et al.* 2003), but never to
 394 the same extent as in the perennial eusocial Hymenoptera such as ants, honeybees and
 395 stingless bees. Our results on leaf-cutting ants and comparisons with other eusocial
 396 insects suggest that there is a fundamental connection between size and longevity of
 397 eusocial colonies, prophylactic disease management, and the longevity and robustness
 398 of males, the sex that is increasingly reduced to (and specialized on) semelparously
 399 transferring sperm as eusociality becomes more advanced.

Acknowledgements

We thank Louise Lee Munk, Nicolai Vitt Meyling and Jørgen Eilenberg for making the *Metarhizium brunneum* plates available, David Nash for statistical help, Susanne den Boer and Gaspar Bruner for helping to collect ants in the field, the Smithsonian Tropical Research Institute for facilitating our field-work, and the Autoridad Nacional de Ambiente (ANAM) for issuing collection and export permits. The work was supported by a grant from the Danish National Research Foundation to JJB (DNRF57) and a Future Fellowship and Linkage Grant from the Australian Research Council to B.B.

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Figure 1

Average survival curves of males from the six *At. colombica* and three *Ac. echinator* 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. echinator* (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. echinator* (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.

Table 1

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 echinator*. *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.

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A

Dependent variable	Model	N	Factor	DF	F	P
Logit sperm viability	Reduced model	224	Species	1, 1.01	0.551	0.592
		224	Year (Species)	1, 5.45	53.25	0.0005
		224	Colony (Year & Species)	6, 211	2.230	0.0416
		224	<i>Metarhizium</i> infection	1, 211	17.53	< 0.0001
		224	<i>Metarhizium</i> infection x Species	1, 211	0.036	0.849
		224	Days after inoculation	1, 211	2.751	0.099
		224	Days after inoculation x Species	1, 211	8.629	0.0037
		Full model	Full model	200	Fresh weight	1, 186
190	Encapsulation response			1, 175	0.176	0.676
190	Fresh weight x <i>Metarhizium</i> infection			1, 174	1.255	0.264
190	Days after inoculation x <i>Metarhizium</i> infection			1, 173	0.755	0.386
190	Encapsulation response x <i>Metarhizium</i> infection			1, 172	0.915	0.340
190	Fresh weight x Species			1, 171	0.407	0.525
190	Encapsulation response x Species			1, 170	0.036	0.850

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Dependent variable	Model	N	Factor	DF	F	P
Encapsulation response	Reduced model	213	Species	1, 1.04	4.82	0.265
		213	Year (Species)	1, 5.80	4.74	0.074
		213	Colony (Year & Species)	6, 203	6.04	< 0.0001
		213	<i>Metarhizium</i> infection	1, 203	0.010	0.921
	Full model	213	Days after inoculation	1, 202	2.551	0.112
		190	Fresh weight	1, 178	2.078	0.151
		190	Days after inoculation x <i>Metarhizium</i> infection	1, 177	1.074	0.302
		190	Fresh weight x <i>Metarhizium</i> infection	1, 176	1.252	0.265
		190	Days after inoculation x Species	1, 175	0.871	0.352
		190	<i>Metarhizium</i> infection x Species	1, 174	0.167	0.684
		190	Fresh weight x Species	1, 173	0.003	0.954

Figure 1

Atta colombica

Acromyrmex echinator

Figure 2

Figure 3