RAQUEL GONTIJO DE LORETO

DISEASE RISK AND FORAGING IN *Camponotus rufipes* (FORMICIDAE, FORMICINAE)

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Magister Scientiae*.

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Co-orientador

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"Toda a nossa ciência, comparada com a realidade, é primitiva e infantil - e, no entanto, é a coisa mais preciosa que temos." (Albert Einstein)

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RESUMO

LORETO, Raquel Gontijo de, M.Sc., Universidade Federal de Viçosa, junho de 2011. **Disease risk and foraging in *Camponotus rufipes* (Formicidae, Formicinae)** Orientador: Simon Luke Elliot. Co-orientadores: José Henrique Schoereder e Og Francisco Fonseca de Souza

Parasitas afetam seus hospedeiros de variadas maneiras, dentre efeitos indiretos até manipulação do hospedeiro. Em insetos sociais, as relações parasita-hospedeiro são mais complexas, pois viver em grupo tem implicações severas ao parasitismo. Assim, reconhece-se que os parasitas representam importante pressão seletiva sobre os grupos sociais. O fungo *Ophiocordyceps unilateralis sensu lato* é um patógeno de formigas restrito a tribo Camponotini. Esse parasita, matador obrigatório, manipula a formiga hospedeira para que sua morte ocorra em local específico, que eleva o *fitness* do parasita. Muitos trabalhos têm mostrado o quanto esse sistema é otimizado para o parasita, mas pouco estudo tem sido direcionado para as formigas hospedeiras. Nós mostramos que em condições naturais, a formiga *Camponotus rufipes*, hospedeira do *O. camponoti-rufipedis* usa objetos previamente existente no seu habitat, como cipós, gravetos e galhos caídos, como pontes para construir suas trilhas de forrageamento, as quais são mantidas ao longo do tempo como trilhas-tronco. As pontes funcionam como vias expressas, sobre as quais as operárias gastam menos tempo para se deslocar, mesmo usando caminhos mais longos. Assim, diferente do que tem sido considerado até então, otimizar o forrageamento não significa usar o caminho mais curto. Se o fungo pode manter total controle sob as hospedeiras infectadas, esperaríamos que as formigas morressem próximas as trilhas, que representam fontes de novos possíveis hospedeiros. No entanto, nós encontramos que as formigas mortas pelo fungo morrem aleatoriamente em relação às trilas de forrageamento. Apesar da existir a possibilidade das trilhas estarem na área de alcance dos esporos do parasita, o uso das pontes em trilhas de forrageamento parece diminuir o contato das forrageadoras com o solo da floresta. Nós sugerimos que, apesar da manipulação, a hospedeira é capaz de reagir a infecção, protegendo sua colônia.

ABSTRACT

LORETO, Raquel Gontijo de, M.Sc., Universidade Federal de Viçosa, June, 2011. **Disease risk and foraging in *Camponotus rufipes* (Formicidae, Formicinae)** Adviser: Simon Luke Elliot. Co-advisers: José Henrique Schoereder and Og Francisco Fonseca de Souza

Parasites have effects on their hosts in different ways, range from indirect effects to manipulation of host. In social insects, the parasite-host relationship are more complex once living in group has consequences on parasitism. Then, it is known the parasites represent a great pressure over social insects. The pathogenic fungi *Ophiocordyceps unilateralis sensu lato* is an ant pathogen limited to Camponotini tribe. This parasite, obligate killer, manipulates the ant host leading her to die in a specific place, where the fungi growth is greater. Many studies have shown that the system describe above is much optimized for the fungi, but little attention has been focus on the ant hosts. We show, in natural conditions, the ant host *Camponotus rufipes*, of *O. camponoti-rufipedis*, uses previous objects on its habitat, e.g. lianas, twigs and fallen branches, as bridges to build the forage trail, which are permanent across the time, characterizing trunk-trails. The bridges are used as high-ways, over which the ants spend less time to move, even using longer way. Thus, different from what has been consider up to now, to optimize the forage does not mean using the shortest way. If the fungus is able to totally control the host, we would expect the dead ants dying closer to trails forage, which are the source of new possible hosts. Nonetheless, we found the mycosed ants die randomly in respect the host forage trails. Although we recognize the possibility the trails could be on parasite spore range, the bridges on seen to increase the host contact with forest floor. We suggest that, even being manipulated, the host may react against the infection, maybe protecting their nestmates.

REVISÃO DE LITERATURA

Ant foraging

Ants (Hymenoptera, Formicidae) occur in all terrestrial habitats except the poles, and they are the dominant members of the communities they occupy. They inhabit a large variety of niches as being direct herbivores, indirect herbivores and predators (Hölldobler & Wilson 1990). However, most species of ants are generalist omnivores.

Generally, a foraging ant starts to search for food by moving constantly far way from the nest. Subsequently, she changes direction until she finds a resource (Traniello 1989). Once food is found, the ants may transport it to the nest. Although some ants forage solitarily, others do so in groups. The strategies to show and to lead the nestmates until the food vary, depending on each species (Traniello 1989), but most species use chemical trails to do so, for example *Solenopsis saevissima* (Wilson 1962); *Camponotus rufipes* (Jaffe and Sanchez 1984); *Atta cephalotes* (Evison et al. 2008). When an ant scout finds food, she returns to the nest laying a droplet of pheromone on the substrate upon which she is walking (Wilson 1962). Inside the nest, ants alert and recruit their nestmates by pheromone or direct contact (Hölldobler & Wilson 1990). The recruited nestmates follow the pheromone cues to the food and, if the recruited ants have success feeding, they return to the nest laying more pheromone cues on the substrate (Wilson, 1962). Thus, a pheromone trail is created by positive feedback. Over the positive feedback system of foraging trail, the shortest route has the highest probability of being reinforced in a determined period of time compared to alternative routes. Ants are thereby able to optimize their foraging trail (Buhl et al. 2009, Reid et al. 2011). By similar processes of resource evaluation and trail reinforcement, they are able to concentrate on higher quality resources (Beckers et al.

1990). Associated with the division of labour, this foraging efficiency has been used to explain the dominance of ants biomass compared with other insects (Bourke et al. 1995, Wilson & Hölldobler 2005).

Insect-pathogen interactions

Insect-pathogen interactions are quite diverse (Bonsall 2004), and may have direct and indirect consequences on the insect host. A common outcome of disease is the death of the host. Nevertheless, many other sublethal or indirect effects arise from disease, such as alterations in behavior (Orr 1992, Elliot et al. 2002, Bonsall 2004, Andersen et al. 2009), coloration (Fenton et al. 2011), food intake, development (Nathan et al. 2006, Roy et al. 2006) and susceptibility to secondary predation or parasitism (Arthurs e Thomas 2001, Ban et al. 2008).

With respect to eusocial insects, the fact that they live in groups can complicate the host-pathogen interaction. It is expected that pathogen transmission among these individuals by direct contact is more frequent than in solitary insects (McCallum et al. 2001). Thus, the necessity of information transmission and all life stages occurring inside the nest represent an increased dispersion and different habits to be explored by parasites (Schmid-Hempel 1998).

On the another hand, social insects have many defenses that minimize the chances of success of pathogens. Schmid-Hempel & Ebert (2003) refer to a “cascade of defense components” which would be composed of different resistance mechanisms, acting in different levels, sequences and specificities. According to these authors, there are behavioral, physical or physiological and immunological mechanisms of defense. In addition, some characteristics of social insects, such as division of labor, make them able to minimize the diseases (Cremer et al. 2007). The division of labor as a behavioral mechanism of defense is consistent with the conveyor belt model proposed by Schmid-Hempel (1998). According to him, younger workers remain inside the nest but as they age they go outside the nest. There they undertake activities such as foraging and defense that carry greater mortality risks, as they have become a less valuable class of workers. Thus, age polyethism is a central feature of the model. These older workers are assumed never to come back closer to the queen and the brood of colony, and therefore this division increases the life span of the individuals and of the colony. Empirical data reinforce this as leaf-cutting ant foragers are less resistant to an entomopathogenic fungus than the workers inside the nest (Fellet et al. in prep.).

To an obligatory pathogen, these defenses can result in a coevolutionary "arms race" which may affect the frequency of resistant genotypes in a population (Jaenike 1978, Hamilton 1980) and favor selection for genetic diversity in a colony of hosts, as has been showen for some insect societies (Hunt & Page 1995, Sirviö et al 2006). Because of this, parasites are known to impose selective pressure on social organization, affecting diverse attributes from the behavior of the colony to its genetic life history (Feener 1988, Schmid-Hempel and Schmid-Hempel 1993, Keller 1995, Orr et al. 1997, Rosengaus and Traniello 2001). There are many recordings of behavioral changes following invasion by parasites, including increased grooming and nest cleaning, inactivity inside the nest and removal of infected and dead individuals (Meikle et al. 2005, Roy et al. 2006, Wilson-Rich et al. 2007). Such changes in behavior are generally seen as defenses against the parasite. However, there are a number of examples in non-social insects where parasites do more than induce defensive behavioral changes: they manipulate their hosts' behavior, thereby increasing their own fitness (Thomas et al 2002, Grosman et al. 2008).

Ophiocordyceps unilateralis: extended phenotype

Insect-entomopathogenic fungi probably have an important role in regulating insect population dynamics in tropical forests (Evans & Samson 1982). The life cycles of these organisms are synchronized with their host stages and environmental conditions (Shah & Pell 2003), and most of them need to kill their host to complete the cycle. The hosts of entomopathogenic fungi become infected after the spores penetrate the external cuticle of the insect skeleton. Inside the insect body, in the haemolymph, the fungi produce yeast-like cells that grow and spread throughout the insect acquiring nutrients. It is probable that fungi use different strategies to kill their hosts, some causing host death by physiological starvation and others by producing toxins (Kershaw et al. 1999). Following host death, the fungus must penetrate the insect cuticle again, this time from the inside out, to produce propagules outside the host body that are able to infect other hosts, completing the cycle (Samson et al. 1988).

The genus *Ophiocordyceps* (Hypocreales, Claviciptaceae) consists of more than 150 species of entomopathogens that infect different arthropod groups (Sung et al. 2007). *Ophiocordyceps unilateralis,* formerly known as *Cordyceps unilateralis,* infects ants of the Camponotini tribe (Evans 2003), such as *C. rufipes*. This fungus is a common ant pathogen that is usually registered in the tropics and subtropics, with sporadic records in temperate habitats.

The interaction of *O. unilateralis* with its host is an extraordinary example of host manipulation which leads the infected host ant to a very precise location before it is killed.

The infected host ant, when in this appropriate locale, bites the underside vein of a leaf, on the north-northwest side of the plant, at approximately 25 cm from the ground. This active fungal positioning of host in the well-defined manipulative zone can be considered an extended phenotype and it increases the fitness of the fungus. This interaction is particularly interesting because the ant host nests in the canopy and its trails are concentrated in the canopy, yet the fungal spores create an infectious “killing field” on the ground, where it is dispersed over short distances (Andersen et al. 2009).

In an evergreen primary forest in Thailand, *O. unilateralis* infects the host *C. leonardi* (Andersen et al. 2009, Pontoppidan et al. 2009). This ant host has a canopy nest and trails and they die closer to the ground than their natural habitat (the canopy). In Brazil, this fungus occurs both in primary and secondary forest (Evans & Samon 1984). Recently, four new species of *Ophiocordyceps* were described in the state of Minas Gerais, Brazil, and each new species has a different specific ant host (Evans et al. 2011). This dissertation focuses on a recovered fragment of Atlantic rainforest (previously a coffee plantation), in Viçosa, Minas Gerais, Brazil, where the most common host ant is *Camponotus rufipes* and its pathogen is *Ophiocordyceps camponoti-rufipedis*.

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INTRODUÇÃO

Introdução geral

Estudos sobre interações entre insetos e patógenos vêm apresentando grande desenvolvimento nos últimos anos, desde pesquisas básicas sobre a dinâmica do patógeno ou hospedeiro, até investigações de uso de agentes microbianos para o controle biológico de pragas (Thomas et al. 2003). A ecologia das interações inseto-patógeno é bastante diversa (Bonsall 2004), tendo consequências diretas e indiretas sobre o inseto hospedeiro, sendo que o principal resultado dessa interação é a sua morte. No entanto, muitos outros efeitos indiretos oriundos dessa relação são discutidos, como: mudanças comportamentais (Elliot et al. 2002, Bonsall 2004), alteração do consumo alimentar, atividade e estágios de desenvolvimento do inseto (Nathan et al. 2006, Roy et al. 2006, Hernández-Velázquez et al. 2007) e aumento da susceptibilidade à predação e parasitismo (Arthurs e Thomas 2001, Ban et al. 2008).

Uma importante questão para o parasita é como ele irá encontrar novos hospedeiros. Os parasitas utilizam diferentes estratégias de transmissão para completar seus ciclos de vida. Aqueles que não possuem formas infectivas móveis transmitidos podem ser transmitidos passivamente via água da chuva (Bruck & Lewis 2002) ou vento (Aylor 1990) e, aleatoriamente, encontrar um novo hospedeiro. Alguns parasitas liberam estágios ativos, os quais encontrarão ativamente um novo hospedeiro. Por exemplo, nematóides entomopatogênicos do gênero *Heterorhabditis*, que são matadores obrigatórios (Ebert & Weisser 1997), matando seu hospedeiro para completar o ciclo de vida, liberam um formas infectivas de vida livre, que irão encontrar um novo hospedeiro (Kaya & Gaugler 1993). Entretanto, quando parasitas matadores obrigatórios não possuem forma propagativa móvel, como os fungos entomopatogêncios, o local no qual o hospedeiro morre pode ser muito importante para a transmissão da doença a novos hospedeiros. Nesse contexto, uma eficiente estratégia é manipular o hospedeiro e garantir que este morra em local adequado (McCurdy et al. 1999, Thomas et al. 2002), que otimiza o *fitness* do parasita manipulador.

Um exemplo de manipulação de hospedeiros é o sistema *Ophiocordyceps unilateralis sensu lato* e formiga hospedeira. O fungo parasita *Ophiocordyceps* controla o comportamento da formiga infectada, conduzindo a hospedeira até uma localização precisa antes que ela morra. Na Tailândia, formigas da espécie *Camponotus leonardi*, infectadas pelo fungo, morrem à 25cm do solo da floresta, onde a esporulação do fungo é máxima (Andersen et al. 2009). Também se sabe que as formigas infectadas pelo *O. unilateralis* encontram-se agregadas (Pontoppidan et al. 2009). Mesmo que o fungo conduza a formiga até uma localização específica, onde se crescimento é elevado, se os esporos não alcançarem formigas vivas sadias, o ciclo do fungo não se fechará. Como as formigas vivas geralmente se distribuem no espaço seguindo trilhas de forrageamento, essas trilhas representam para o fungo fonte de novos possíveis hospedeiros. Nesse contexto, consideramos que seja importante entender a ecologia do comportamento de forrageamento das formigas, antes de estudar a interação entre parasita e hospedeiro.

No primeiro capítulo da dissertação, nós concentramos os estudos no comportamento de forrageamento da formiga hospedeira. Observações preliminares mostravam que as trilhas de forrageamento formigas carpinteira (*Camponotus rufipes*; Hymenoptera: Camponotini) seguiam troncos caídos, cipós e gravetos, usando esses objetos como pontes para construírem as trilhas. Como as pontes pareciam tornar as trilhas mais longas, nós hipotetizamos que a rota mais longa, que inclui pontes, é o caminho mais otimizado. Assim sendo, esperávamos encontrar maior permanência, ao longo do tempo, das partes da trilha que passavam por pontes em relação aquelas que passavam pelo solo. A partir de nossos resultados, nós concluímos que, em condições naturais, as formigas otimizam suas rotas não usando o caminho mais curto, e sim o caminho mais rápido. As pontes tornam o caminho mais longo, mas nós estimamos que as formigas gastem 2,4 vezes menos tempo para chegar a um determinado ponto, usando as pontes. Nós também concluímos que as pontes são mantidas ao longo do tempo, caracterizando trilhas-troncos.

Após entender melhor sobre o forrageamento da formiga hospedeira, focamos em estudar a distribuição das formigas mortas infectadas no espaço. Conforme os estudos com *Ophiocordyceps unilateralis sensu lato* tem mostrado a capacidade de manipular precisamente a formiga hospedeira, nós esperávamos encontrar as formigas mortas próximas as trilhas de forrageamento, que representa fonte de novos possíveis hospedeiros. Mostramos que as formigas mortas pelo fungo encontram-se aleatoriamente distribuídas em relação às trilhas de forrageamento. Entretanto, a altura média das formigas mortas é maior que as da trilha e, pode ser que as trilhas estejam na área de cobertura dos esporos.

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CAPÍTULO 1:

Foraging ants trade off further for faster: use of natural bridges and trunk-trail permanency in carpenter ants

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Nota: o Capítulo 1 foi escrito de acordo com as exigências do periódico *Animal Behaviour*.

Foraging ants trade off further for faster: use of natural bridges and trunk-trail permanency in carpenter ants

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The self-organized nature of many biological networks, where solutions to problems emerge through the interaction of relatively simple "agents", has potential for bio-inspired solutions to similar problems in the human world. In particular, the complex foraging behavior exhibited by trail-making ants inspired the creation of Ant Colony Optimization algorithms (ACO) that can provide great insight to diverse problems. Up to now, the ant foraging optimization has been based on using the shortest way to construct the trail, and most of these results were obtained in laboratory conditions. We showed that carpenter ants, in natural conditions, optimize their foraging trail not using the shortest way, but the faster way. The ant *Camponotus rufipes* uses previous fallen branches, twigs and lianas as bridges, to build their trails. The bridges make the walk longer than if they walk in a straight line through the forest floor, but we estimated the ants spend 2.4 less time to reach the same point following the bridges. Also, we found the bridge trails are maintained for a long time, characterizing trunk-trails.

Studies of the organization of complex animal societies can provide direct insight into the evolution of social behavior in higher organisms. Such studies can also provide insight into the major transitions of evolution, in particular the evolution of multi-cellular life from interacting groups of more-or-less cooperative single cells (Smith & Szathmary 1997). However, the self-organized nature of many biological networks, where solutions to problems emerge through the interaction of relatively simple "agents", has potential for bio-inspired solutions to similar problems in the human world. For example, the complex foraging behavior exhibited by trail-making ants inspired the creation of Ant Colony Optimization algorithms (ACO) that can solve (or at least provide great insight into) diverse problems including telephone networks (Zhao et al. 2010) and delivery schedules (Lee 2009).

In pheromone-based ant trail networks (such as those found in *Camponotus rufipes* (e.g. Jaffe & Sanchez 1984), *Solenopsis saevissima* (e.g. Wilson 1962) and *Atta colombica* (e.g. Evison et al. 2008), ants follow a chemical foraging trail laid by nestmates to a food source. If food is successfully foraged at the site, returning foragers will lay further pheromone, reinforcing the trail, which is constantly decaying through evaporation. If following the trail from the nest does not result in food then returning foragers will not reinforce it and the trail will rapidly decay beyond the point that it is attractive to potential ant followers (Hölldobler & Wilson 1990). Within this organizational framework, shorter trails to a resource will tend to prevail over longer trails because shorter trails are reinforced at a greater rate against a background of constant pheromone evaporation. This positive feedback, linked with trail evaporation, also underpins ACO algorithms (Dorigo 1992), albeit in a more abstract sense.

Many studies have supported the hypothesis that ant colonies can optimize foraging by, for example, choosing the best resource (Beckers et al. 1990). Ants can also maximize forage return by creating an alternative trail route in crowded situations (Dussutuor et al. 2004) and are able to find the shortest route in complex dynamic situations (Reid et al. 2011). Other studies have also demonstrated the importance and adaptive significance of efficient decision making at trail bifurcations, which are typical of ant foraging networks (Jackson et al. 2004, Vittori et al. 2006, Garnier et al. 2009). However, a particular question that has not been addressed is how ants adapt to differences in the terrain over which they travel. Furthermore, pheromone studies have mostly been carried out in the laboratory and are therefore severely limited with respect to the insight they can offer to ant foraging under real, and complex, ecological scenarios.

Very few studies have examined trail optimization in field conditions. One study that has (Buhl et al. 2009) showed that the foraging trail network of the wood ant *Formica aquilonia* optimizes the total length of trail and the distance between the source and nest. Ant species that forage on the forest floor, like *F. aquilonia*, have a complex three-dimensional foraging environment, with fallen branches, trees, lianas and roots forming both obstacles and potential walkways. Such opportunist walkways may be useful to ants; trails of the leaf-cutting ant *Atta cephalotes,* for example, are composed of ca. 30% fallen branches in one site, and foragers walked faster on the fallen branches than on soil (Farji-Brener et al. 2007). However, branches are unlikely to be aligned with the shortest distance to the destination. If they allow faster walking speeds, though, they may provide the quickest route. Branches and other potential walkways are, therefore, an important but common complication to foraging trails, and can be considered analogous to road networks where the quality of the road surface – dirt tracks versus asphalt, for example – can be an important factor in deciding the best route.

We set out to investigate how ants use bridges in a field setting, in particular whether they use bridges that are not aligned with their trails. Preliminary observations showed that the foraging trails of carpenter ants (*Camponotus rufipes*; Hymenoptera: Camponitini) followed fallen branches, twigs and lianas, using these bridges as part of the trail. As this seems to make their trails longer, we hypothesized that the longer route, where it includes these bridges, is the optimal route; that is, the ants are trading off "further" for "faster". If this is the case, then we expect to see a consistency in the pattern of use of these bridges through time, as is seen in leafcutting ant trunk trails (Farji-Brener et al. 2007); thus, our second hypothesis is that trails will be longer-lived when on bridges than when on the forest floor.

METHODS

Study area and species

Fieldwork was carried out from December 2010 to April 2011 at the Mata do Paraíso research station of the Federal University of Viçosa, Minas Gerais, southeastern Brazil (20°48'08 S 42°51'31 W). This is a recovered fragment of Atlantic rainforest (it was previously a coffee plantation) where the dominant vegetation is a secondary seasonal semi-deciduous montane forest (Veloso et al. 1991). Although it is characterized by a robust understory, foliage is not too thick at ground level. The ground is typically covered with 10-20cm of leaf litter, though there are patches where soil is exposed. *Camponotus rufipes* is very abundant in this fragment, feeding on nectar and honeydew as well as live arthropods (Jaffe & Sanchez 1984, Del-Claro & Oliveira 2000). In this habitat, *C. rufipes* is active at night, with activity peaking in the early evening (Figure S1, Supplementary material).

Data collection

Four established nests of *C. rufipes* were identified. These were approximately 1-2m in diameter and were built on the forest floor against living trees. A square plot (10m x 10m) was demarcated around each nest, such that the nest entrance was central. Each plot was sub-divided into a 25-square grid (each grid square being 2m x 2m) and foraging trails were marked with small flags placed every 30cm, starting at the nest and continuing until trails left the plot. This was done shortly after dusk, when foraging peaked (Figure S1, Supplementary material). Different colored flags were used to identify those parts of the trail following bridges (fallen branches, twigs and lianas) and those occurring on the forest floor. The coordinates of each flag inside the plot were determined by measuring the x and y position. The trails were flagged and monitored in this manner once a month for four months (December to March).

To record trail activity, trails were filmed between 7.30pm and 8.30pm on the 23rd of March. We chose this time because it is when the ants are most active (Fig S1). A hand-held microscope (ProScope HR - Bodelin Technologies, Lake Oswego) was used at a magnification of 10X and a distance of 15cm from the trails. It was attached to a notebook computer and films were recorded as .avi files. Illumination was red light (a hand-held torch – ShyLux Km-8305 – covered with red electrical isolation tape). Two such recordings were taken from three of the plots, one recording on bridges and one on the forest floor (giving a total of six films). Each recording was 5 minutes but as it was necessary to touch the trail with a rule to standardize the camera distance, the first minute of each recording was excluded from all analyses to allow for ants to recover from any disturbance.

Almost the analyses were conducted in the free software R (version 2.12.0). The exceptions were specified on text.

Do ants use bridges more than the forest floor?

We wished to test our observation that ants were using bridges more than the forest floor. For each monitoring event in each plot, we calculated the number of bridge flags and soil flags for each trail. We compared the number proceeding an ANOVA test via Generalized Mixed. A mixed model was utilized to avoid temporal pseudo-replication and each plot was the replication. Model simplification was carried out to determine the significance of each variable (Crawley 2007). The initial complete model had month and place (bridge or forest floor) as explanatory variable and number of flag as a response variable.

Do ants trade off "further" for "faster"?

We hypothesized that the ants trade off "further" for "faster" on their trails. To test this we did two different analyses. Firstly, to see if they are walking further than strictly necessary, we compared the total trail length from nest entrance to plot edge with the theoretical minimal possible distance they could walk to arrive at the same point. The minimal possible distance was calculated using trigonometrical rules. The actual trail distance was calculated by multiplying the number of flags on the trail by 30cm. Over the four months of study, and in the four plots, we recorded in this manner 21 newly-formed trails that led from nest to plot edge (i.e. any trail that was recorded in consecutive months was used only once in this analysis). Here, then, our repetitions are trails rather than plots, independent of the month in which they were recorded**.** The each actual trail distance was compared with the corresponding minimal theoretical distance.

To test the hypothesis that ants walk faster on bridges than on the forest floor, we compared the speeds of ants walking on each substrate. Speed was measured using Observer® XT software (Noldus, www.noldus.com). This plays recordings in slow motion and users can record behaviors and duration by pressing specific keys sequences. For the first 20 ants that appeared on the screen, for each video, the speeds were recorded. In total, 60 ants for bridges and 54 for forest floor were analyzed (in one colony, only 14 ants were recorded in the four minutes of observation). The speeds were analyzed with a two-way ANOVA in R (version 2.12.0). The response variable was speed (cm sec^{-1}), with a Gamma distribution to account for non-homogenous distribution (Crawley 2007). The explanatory variables were trail substrate (bridge *vs*. forest floor) and the colony of origin of the ants. Individual ants were considered replicates.

We wished to estimate the total time spent using each trail, consisting of bridge and forest floor, and compare it with a hypothetical trail that ran only on forest floor. To obtain the first value, we added (a) the product of the length of the trail on the bridge and the mean speed of ants from that colony on bridges (see above), to (b) the product of the length of trail on the forest floor and the mean speed on that substrate. Estimates for the direct route were taken using the direct distance to the edge multiplied by speed on the forest floor. These paired values were compared with a one-tailed paired t-test.

Are trails on bridges longer-lasting than trails on the forest floor?

To test the hypotheses that trails laid on bridges last longer than trails laid on the forest floor, we calculated the Jaccard index (a similarity index) for the two different conditions (bridge and forest floor); this index is commonly used in ant species composition studies, comparing presence/absence of species in different situations (Zelikova & Breed 2008, Gotelli et al. 2011). We did this using the first month as a reference point to compare with the following months. Thus, we considered the presence or absence of each flag that was present in December with presence/absence data from the next three months. The software PAST (version 1.82b) was used to obtain the Jaccard index. The indices were analyzed by ANCOVA via Generalized Linear Models. The similarity index was used as the response variable, using a binomial distribution. The initial complete model had time (in days) and place (bridge or forest floor) as explanatory variables, while nests were used as replicates. Model simplification followed Crawley (2007).

RESULTS

Ants use bridges more than the forest floor

The majority of the ants' trails follow natural bridges (ANOVA mixed model: χ^2_{1}=71.676, $P<0.001$) rather than the forest floor (Figure 1) and this remained unchanged through time (ANOVA mixed model: χ^2_{3}=0, P=1). We found the 22±5.31 (mean±SE) flags on bridges and 2±0.91 (mean±SE) flags on forest floor per trail. It means that approximated 92% of trail is over bridges and in only 8% of trail the ants walk direct on forest floor.

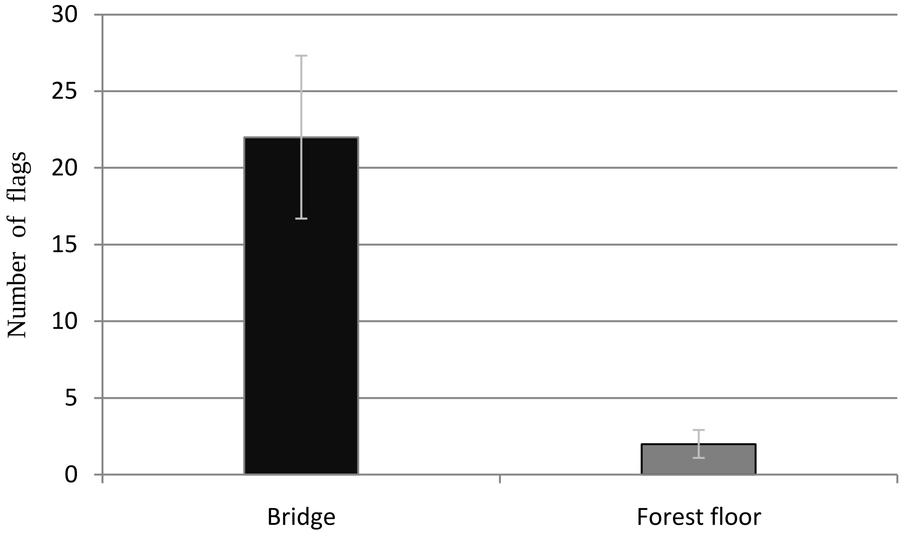

Figure 1: Mean (±SE) of flags used to follow trails of the carpenter ant *Camponotus rufipes* on bridges (fallen branches, twigs and lianas – black bar) and the forest floor (litter – gray bar), in four colonies located in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, southeastern Brazil. Around each nest (N=4) an area of $100m^2$ was delimited and the trails were marked with flags each 30cm, starting on nest entrance until the edge of the delimited area. This was done from December 2010 to March 2011, making four months of observations.

Ants trade off "further" for "faster"

Although ants seem to prefer following bridges, this means they walk further than necessary, when we compared actual trail length (692.92±152.69 cm (mean±sd), *N*= 20) with the theoretically shortest trail (564.52±67.16 cm (mean±sd), *N*= 20) (t-test: t_{19}=3.7737, *P*<0.001) (Figure 2).

The speeds of the workers were influenced both by the substrate used (bridge or forest floor) (two-way ANOVA with Gamma distribution: χ^2_{113}=38.369, *P*<0.001). The nest of origin also influenced speed (χ^2_{113}=69.302, *P*<0.001) and there was an interaction between these two variables (χ^2_{113}=31.464, *P*<0.001). Because of the effect of nest and the interaction, we conducted further analyses to determine if the principal result (the effect of bridge on speed) varied with nest of origin; we found that the direction of the result was maintained but the nest of origin only affected the strength of the difference in speed (see Figure S2, Supplementary Material)

Although ants walked further using bridges, they walked considerably faster on bridges (5.54±3.19 cm/s (mean±sd), *N*=60) than on the forest floor (1.58±1.19 cm/s (mean±sd), *N*=54) (ANOVA with Gamma distribution: χ^2_{113}=49.010, *P*<0.001) (Figure 3). Using these speeds in conjunction with the lengths of trail found on bridges or on the forest floor, we estimate that the ants reached the edges of the plots 2.4 times faster than if they had walked the direct minimal route on the forest floor (Figure 4) (t_{18}= -7.5164, *P*<0.001).

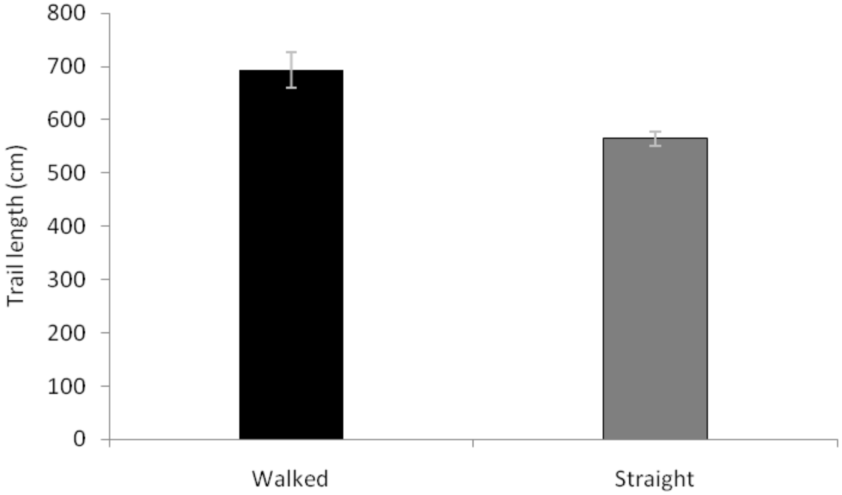

Figure 2: The mean (±SE) of minimal theoretical distance of trail (Straight - gray bar) and mean (±SE) real distance walked by workers of ant *Camponotus rufipes* (Walked – black bar), in centimeter, in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, Southeast Brazil. It was used 21 trails of four different nest of *C. rufipes*. Around each nest was delimited an area of 100m^2. The distance walked was calculated from the nest entrance until the edge of delimited area. The theoretical minimum distance was calculated by trigonometric rules from the nest until the same point of edge of real trail.

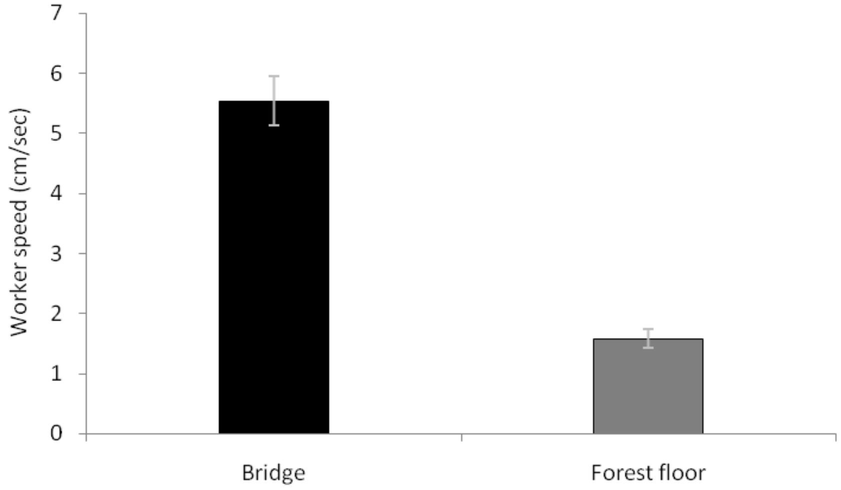

Figure 3: The mean (±SE) worker speed (cm/s) of carpenter ant *Camponotus rufipes* on bridge (fallen branches, twigs and lianas – black bar) and on forest floor (litter – gray bar) at busier time on trail (see Figure S1, supplementary material), in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, Southeast Brazil. We recorded 60 ants walking on bridges and 54 ants walking on forest floor with a hand-held microscope and analyzed the images in appropriate software. The recorded location were three of four area cited above.

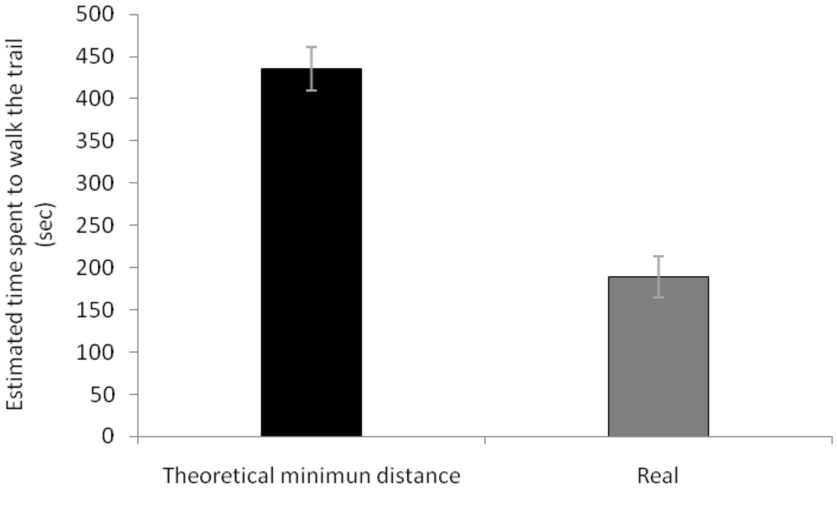

Figure 4: The mean (±SE) estimated time spent by a worker ant *Camponotus rufipes* to walk the trail using the theoretical minimum distance through the forest floor (black bar) and the real distance they walk on natural condition (gray bar), in centimeter, in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, Southeast Brazil. It was used 19 trails of three different nests of *C. rufipes*. Around each nest was delimited an area of $100m^2$. The distance walked was calculated from the nest entrance until the edge of delimited area. The theoretical minimum distance was calculated by trigonometric rules from the nest until the same point of edge of

real trail. We used the speed relative for each colony to estimate the theoretical and the real time spend.

Trails on bridges do not last longer than trails on the forest floor.

Contrary to our hypothesis, there was no difference in the permanency of trails on the different substrates (ANCOVA: $F_{[1,31]}$=0.3949, *P*=0.5297). The trail permanence depends only of days ($F_{[1,31]}$=12.956; *P*<0.001). Although our hypothesis was rejected, we did find that trail persistence was considerable. Approximately 50% of the trail is maintained after 60 days and 20% after 80 days (Figure 5).

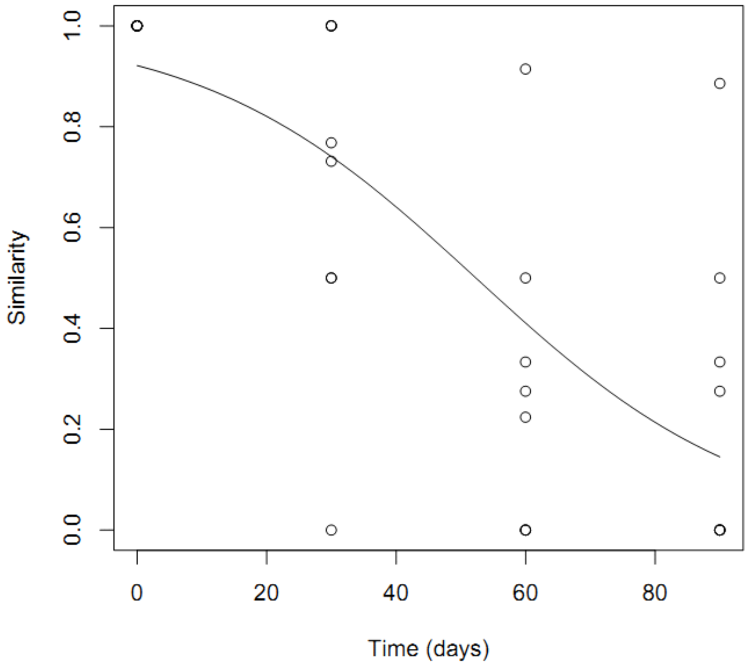

Figure 5: The foraging trail permanence on time (days) of carpenter ants, *Camponotus rufipes*, in Viçosa, Minas Gerais, Brazil. The Jaccard index of similarity was calculated for four colonies of *C. rufipes*, relating the trail of December 2010 with the three next months (January to March 2011). The similarity indices were analyzed in a logistic regression. Approximately 50% of trail was maintained after 60 days and 20% of trail was there in the last observation.

DISCUSSION

Studies of ant foraging universally show (to the best of our knowledge) that ant trails follow the shortest route (e.g. Jackson et al. 2004; Garnier et al. 2009; Vittori et al. 2006; Reid et al. 2011). Here we show an exception to this rule: the optimal trail is not necessarily the shortest. The ant *C. rufipes* uses bridges (fallen branches, twigs and lianas) for most of its trail in preference to the forest floor. Although they walk 1.22 times further, they walk 3.5 times faster on the bridges, and we estimate that this means they spend 2.4 less time walking over the lengths of the real trails, which are combination between bridges and forest floor. Contrary to our initial expectation, however, the portions of trail on bridges are not longer-lasting than those on the forest floor. Probably, the soil trails were under sampled because the ants use mainly the bridges on their trails.

Although the ACO algorithm and its variations have been applied successfully to problem solving (Dorigo 1992; Bullnheimer et al 1999; Iyengar et al. 2007; Ogden & Karaboga 2009), we have uncovered a new feature of trail optimization. We have shown that the carpenter ant *C. rufipes* uses “opportunistic” objects, for example fallen branches, twigs and lianas, as bridges, to build their foraging trails. These bridges are distributed haphazardly in space and are very unlikely to represent the shortest route. Nevertheless, 89% of the ants’ foraging trail is composed of bridges. We have also shown that, by using bridges, the ants can walk faster than walking directly on forest floor. Compiling the two results, we suggest the ants are trading off further for faster, and using the shortest trail is not always the optimal solution.

Ants following fallen branches, twigs and lianas have been reported in previous works. Yamamoto & Del-Claro (2008) report that *Camponotus sericeiventris* uses branches and lianas as part of their trails, but provide no additional information. Further, Farji-Brener et al. (2007) found that 30% of foraging trails laid by the leaf-cutting ant *Atta cephalotes* are composed of those objects which we classify as bridges. These authors considered the advantages of increasing speed without incorporating any costs of building and maintaining a physical trail; such costs are known from leaf-cutting ants that construct trails on the forest floor (Hölldobler & Wilson 1990).

We suggest that using bridges has advantages in addition to increasing speed. Entomopathogenic fungi play an important role in tropical forests (Evans & Samsom 1982), and we suggest the ants could decrease infection rate by using the bridges and avoiding bare soil. The recovered fragment of Atlantic rainforest where we carried out this work has the entomopathogenic fungus *Ophiocordyceps unilateralis* (Hypocreales, Claviciptaceae), a specific parasite of the Camponitini tribe. This parasitic fungus controls the behavior of the ant host, leading the infected host to climb to a specific height and orientation before dying, forming aggregated "graveyards" (Andersen et al. 2009, Pontoppidan et al. 2009). As the spores of this fungus are too large to be dispersed by wind, the forest floor should be a repository for spores (Pontoppidan et al. 2009). Using bridges reduces potential contact between ants and spores and thereby could reduce the probability of infection. Also, conditions on the forest floor may be quite different from the conditions on fallen branches, twigs and lianas and given that fungal spores are sensitive to microclimatic changes (Andersen et al. 2009), the lifespan of spores on bridge may be smaller than on the forest floor. Another way that bridges could decrease infection rate by *O. unilateralis* arises from the geometry of trail bridges and of sporulating fungi. Bridges are often several centimeters above the ground and this reduces the distance to fungus-killed ants (who are positioned at a height). This also reduces the diameter of the "cone of sporulation" arising from infected ants, which makes it less likely that ants on bridges will be infected by this route. It is also possible in some cases that the ant trails using bridges are higher than the dead infected ants, which are generally only 25cm above ground level (Andersen et al. 2009). An additional advantage of using bridges is that the total area of ground that ants contact may be reduced. Walking over clear bridges requires only that ants contact the trail with the tarsi, whereas more "untidy" substrates on the forest floor are likely to force contact across more of the ant's cuticle. Also, as shown by Jeanson et al. (2003), the pheromone trail of the ant *Monomorium pharaonis* decays faster on a newspaper substrate (i.e. a rough surface with absorbent proprieties) than a plastic substrate (smooth and non-absorbent). Litter on forest floor is likely to be both a rougher and a more absorbent surface than bark covered branches and twigs so the pheromone trail of *C. rufipes* may evaporate more slowly on bridges.

We expected that the trail would be more permanent when on bridges than when on the forest floor but this was not the case. When the trail ran over the forest floor, this was over short distances, usually between two bridges. Thus, this substrate may have been underrepresented in our sampling, perhaps leading to the observed result. It would be interesting to examine trail permanency over the forest floor in sites with less bridges. Even rejecting our hypotheses about permanence, we still obtained an interesting result about ants trail: this is the first study that shows the permanence of a *Camponotus* trail network through an extended period of time, in natural conditions. Usually, studies are focused on daily rhythms (Santos & Del-Claro 2009, Yamamoto & Del-Claro 2008). We have used a novel application of the Similarity index to show that *C. rufipes* maintain 50% of their foraging trail after 60 days. The forage trail permanence corroborates with our hypothesis that trails under natural condition are optimized, even though they are not following the shortest route. We have found no evidence in the literature or in our own observations that carpenter ants maintain physical trails by clearing them, as found in other ant groups like in leaf-cutter ants (Vasconcelos 1990) and harvester ants (Azcarate & Peco 2003), but now we know they use bridges as “high-ways” for a considerable time, which means such bridges can be characterized as “trunk-trails” (Hölldobler & Wilson 1990).

Conclusions

We found that the carpenter ant *Camponotus rufipes* optimizes foraging trails, trading off “further” for “faster”. This is a new view of ant foraging optimization, a field that has inspired problem-solving in the computer sciences (Dorigo 1992; Bullnheimer 1999; Iyengar et al. 2007; Ogden & Karaboga 2009). We have also provided new information on an important group of ants, showing that they use previous present objects (bridges) in the forest to construct their foraging trail. These trails are persistent through time so we suggest they are trunk-trails in the same fashion as trunk trails constructed by leafcutter ants.

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Supplementary material

Ant schedule

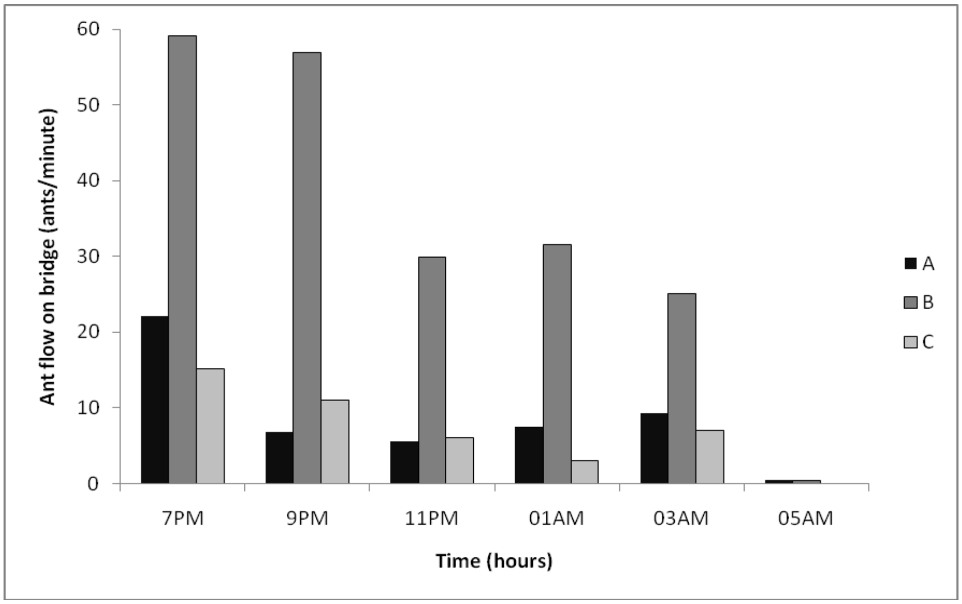

Figure S1: Nighttime foraging activity of *Camponotus rufipes* from three distinct colonies (A,B and C), in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, Southeast Brazil. The data were collected on March 23rd 2011, recording the trail on a bridge path with a hand-held microscope. The locations for recording were chosen according to ease of tripod placement and were maintained throughout the night. The records were made every two hours, starting after sunset, because before this the ants do not forage, and were taken until sunrise (5AM). We filmed five minutes for each colony and finished each set of recording within 1 hour. We considered only bridges because they compose the main part of foraging trail.

Worker speed

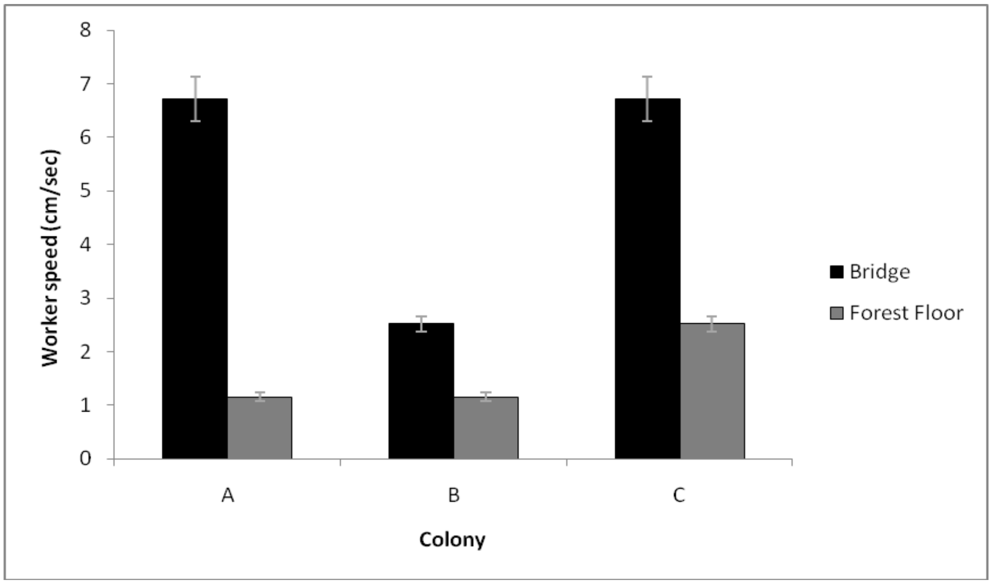

Figure S2: Mean (±SE) speeds of workers of the carpenter ant *Camponotus rufipes* from three colonies (A, B and C) in two diferents parts of foraging trail: Bridges (fallen branches, twigs and lianas) and Forest floor (litter), in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, Southeast Brazil. We recorded 20 ants walking on bridges and 20 ants walking on forest floor, for each colony, with a hand-held microscope and analyzed the images in appropriate software. The films were taken at the busiest time of ant activity (see figure S1). In previous analysis, we found an interaction between colony and substrate (see Methods section). In order to contrast the speed for considering the interaction, we did a new analysis, grouping the nest origin (colony A, B or C) and substrate (bridge or forest floor). In this new analysis our explanatory variables were “trail path + nest” (Bridge colony A, Forest floor colony A Bridge colony B, and so on). The speed of ants of colonies A and B on the forest floor were the same (1.155±0.608 cm/s (mean±SD), N=114) (ANOVA: $F_{[1,109]}$=2.334, P=0.1295). This was slower (ANOVA: $F_{[1,109]}$=34.160, P<0.001) than the speed on the forest floor of workers from colony C and the speed on bridges of workers from colony B (2.518±1.186 cm/s (mean±SD), N=114) (ANOVA: $F_{[1,109]}$=0.0415, P<0.839). Finally, the speeds on the bridge of workers from colonies A and C were the same (6.717±3.195 cm/s (mean±SD), N=114) (ANOVA: $F_{[1,109]}$=0.0824, P<0.5606) and were also the fastest speeds recorded (ANOVA: $F_{[1,109]}$=62.36; P<0.001). Thus, we had already seen that the speeds differed between colonies were different (main text), but with this analysis, we confirmed that the pattern of faster walking speeds on bridges than on forest floor was general, independent of colonies.

Temperatures on bridge versus forest floor

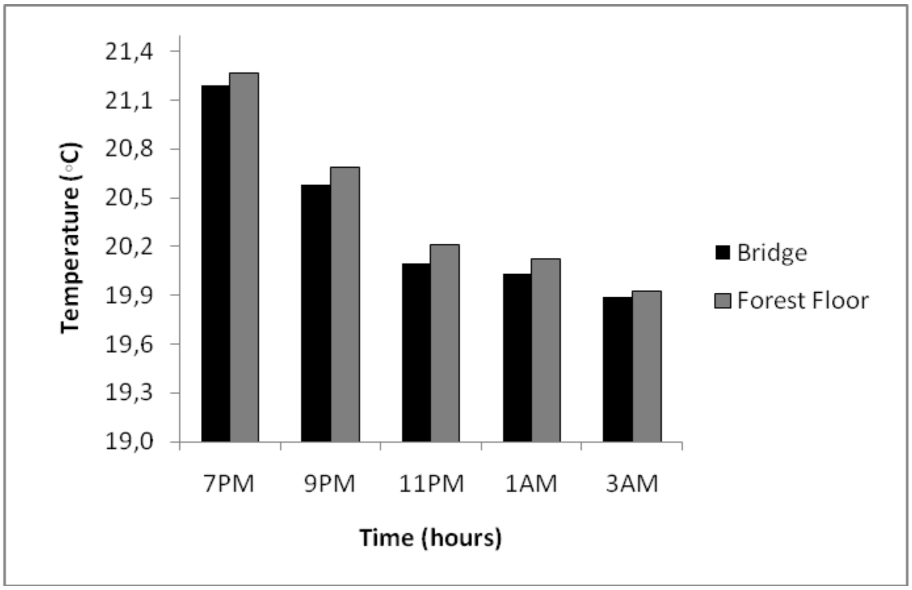

Figure S3: Mean temperatures during the night on ant trails on bridges (black bars) and 30cm from bridges, on the forest floor (gray bars), for three plots (A,B and C)), in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, Southeast Brazil. We recorded the temperature an three points on bridge (and their respective points on forest floor) for each plot. We analyzed the paired measurements using a two-tailed t-test in the free software R. The mean temperature on bridge was very slightly lower (20.347±0.086 (°C) (mean±SE)) than on the forest floor (20.429±0.122 (°C) (mean±SE)) (t_{43}=3.068, p<0.01)

CAPÍTULO 2:

Imperfect parasite manipulation: Zombie ants in space

Raquel G. Loreto; Thairine M. Pereira; Mayara L. R. Freitas;
Simon L. Elliot & David P. Hughes

Nota: o Capítulo 2 foi escrito de acordo com as exigências do periódico *PNAS*.

Imperfect parasite manipulation: Zombie ants in space

Raquel G. Loreto; Thairine M. Pereira; Mayara L. R. Freitas;
Simon L. Elliot & David P.Hughes

A fundamental property of parasites is the method by which they infect new hosts. This is particularly important for parasites that are obligate killers, i.e. that must kill their hosts in order to effect transmission. The place where the host dies may be very important for the parasite transmission and an efficient manner to guarantee a strategic location for host death is to manipulate host behavior. Previous work on the zombie ant system, *Ophiocordyceps unilateralis sensu lato* pathogen and Camponotini tribe ant hosts, documented a precise and fine level of control, with infected workers dying on distinct parts of the leaves, on leaves at a particular height off the ground and orientated at a distinct angle in space. We tested the hypothesis that if the parasite is in total control over the ant, the infected hosts may die close to the ants' foraging trail. We showed, however, that mycosed ants are randomly distributed with respect to the trail. However we found the mycosed ants higher than the ants' trail, possibly bringing the trail within reach of dispersing spores. We suggest that the fungus is not in total control of the host, as the host may be able to defend itself against the pathogen to some degree.

A fundamental property of parasites is the method by which they infect new hosts. Parasites use different strategies to transmit and complete their life cycle. Parasites with non-motile infective forms generally rely on passive transportation via the wind (1) or rain drops (2) and randomly find new hosts. Some parasites release active infective stages, which find a new host actively; for example, insect-pathogenic nematodes of the genus *Heterorhabditis*, which can be considered "obligatory killers" (3) in that host death is a part of their life cycle, but they have a free-living stage that actively searches for new hosts in the soil (4). When parasites are obligate killers but do not have a free-living motile stage (as with entomopathogenic fungi), an alternative strategy to find new hosts is to use the infected host's movement before it dies, to promote contact with a new potential host. Thus, the place where the host dies may be very important for the parasite and an efficient manner to guarantee a strategic location for host death is to manipulate its behavior (5, 6), thus increasing parasite fitness.

We may expect a response from the host, however. When the host is non-social, it may be able to tolerate infection long enough to reproduce before death, so ameliorating the fitness costs of parasitism (7, 8). Another adaptive host response to infection may be to accelerate reproduction before death (9). When we consider social groups of hosts, however, we may see a different pattern of selection pressures on the hosts, and different adaptive strategies. In eusocial insects such as the ants, wasps, bees and termites, where most colony members are sterile and rely on inclusive fitness, there is no possibility to alter investments in life history traits so as to reproduce between infection and death. In this case we would expect the paramount response to be one that protects the group from the parasite

(10), perhaps through behavioral means. When parasitic manipulation of the host is involved, however, this situation becomes much more complex.

The parasitic fungus *Ophiocordyceps unilateralis sensu lato* (Hypocreales, Claviciptaceae) is able to control the behavior of its ant host, leading the infected host to a precise location before killing it. In Thailand, the *Camponotus leonardi* ants die at a specific height (25cm from the forest floor) that maximises fungal sporulation, and with a specific orientation. They are also found in aggregations that have been termed “graveyards” (11, 12). Even with such apparently precise control of host behavior by the parasite, the ant nests and trails are found considerably higher (in the forest canopy) than the height at which infected ants die; this is consistent the idea that ants die in social isolation, so limiting infection of their kin (10). In Brazil, we have essentially the same host-pathogen system but with a peculiar difference: the healthy ants and the fungus are found near to the forest floor. The nests and trails of the carpenter ant *Camponotus rufipes* healthy ants are found on or close to the soil, as a similar height to that of infected dead ants in Thailand (11). The trails are built mainly following bridges (i.e. fallen branches and twigs, and lianas) rather than directly on the forest floor (Chapter 1). We set out to test the hypothesis that manipulation is more optimized for fungus when they are at same space the new possible hosts. If it is true, we expected the height of dead, mycosed ants to be greater than that of the trails. Also, we should find these mycosed ants to be aggregated around the ants’ trails. If we do not find this, on the other hand, we may begin to suspect that infected ants are not under the total control of the fungus, perhaps due to selection pressure to avoid infecting their kin.

Results and Discussion

System description. Figures 1,2,3 and 4 show the data we collected in four sites once per month, during the four study months (December 2010 to March 2011), for *Camponotus rufipes* killed by *Ophiocordyceps camponoti-rufipedis* and ant forage trail.

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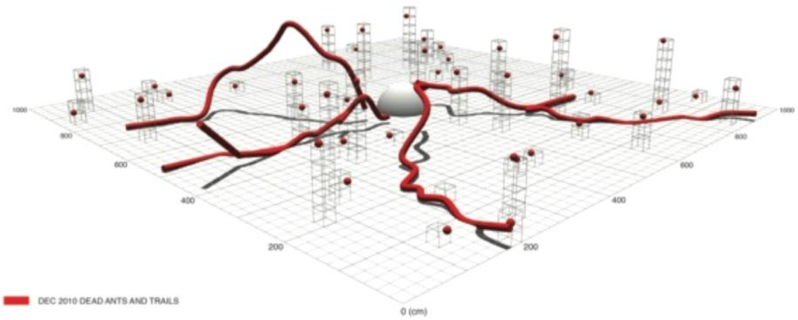
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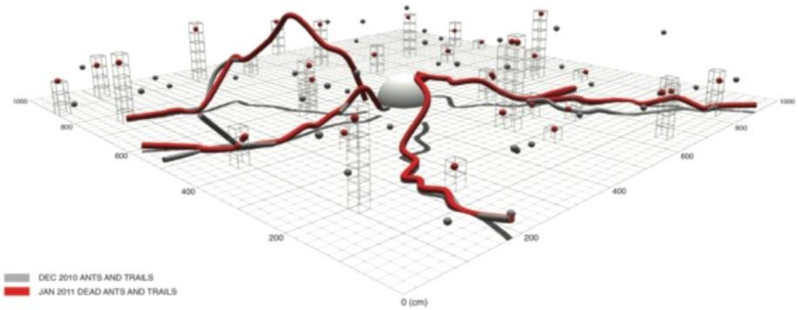
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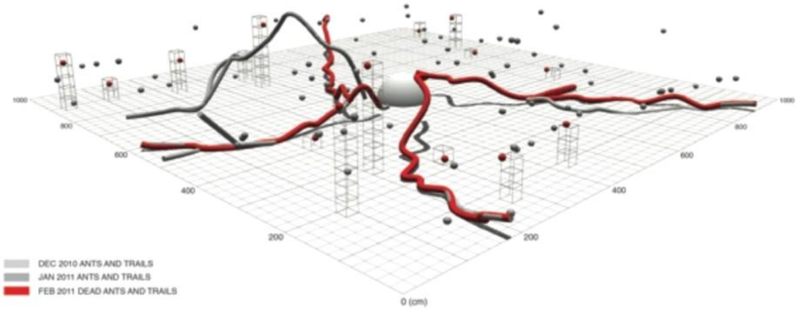
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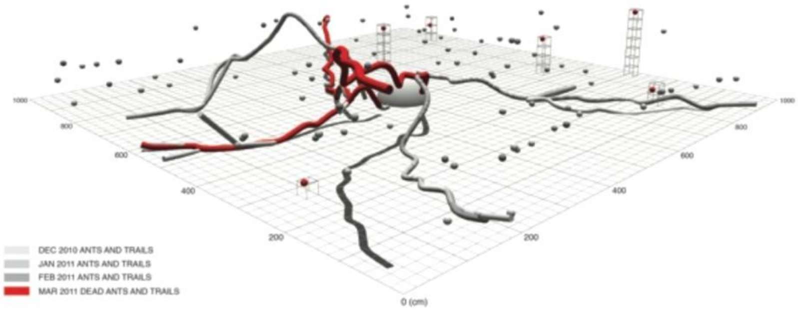
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Figure 1: Distribution of dead *Ophiocordyceps camponoti-rufipedis* infected ants (dots) and foraging trail of host ant *Camponotus rufipes* (lines) across four months around the colony A, in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, southeastern Brazil. (A) December. (B) January. (C) February. (D) March. The red dots are the new dead ants for that month. The red lines are the trails for each month. Where the dots or trails are grey these are ants or trails from the previous month(s). The small squares below the dots (dead ants) show the heights of the ants (each square corresponds to 50cm x 50cm).

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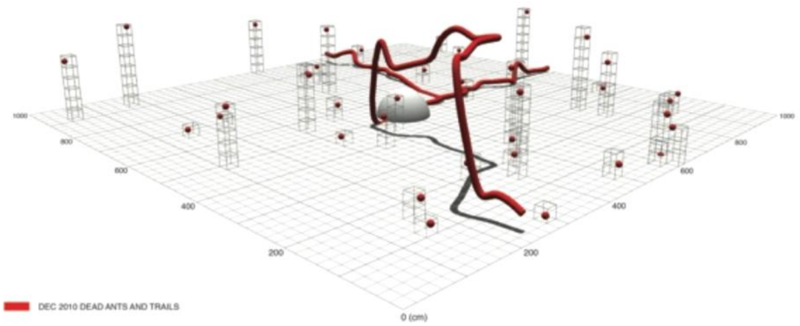
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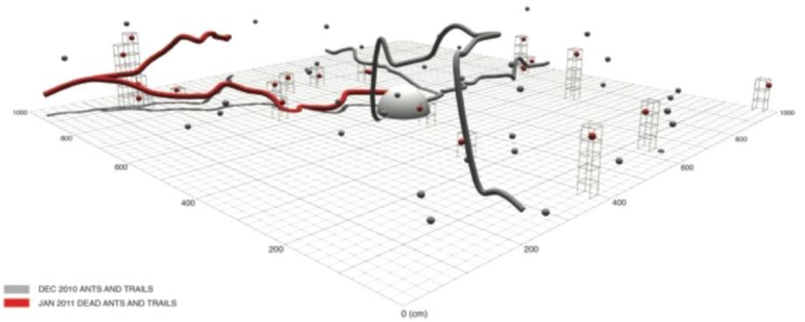
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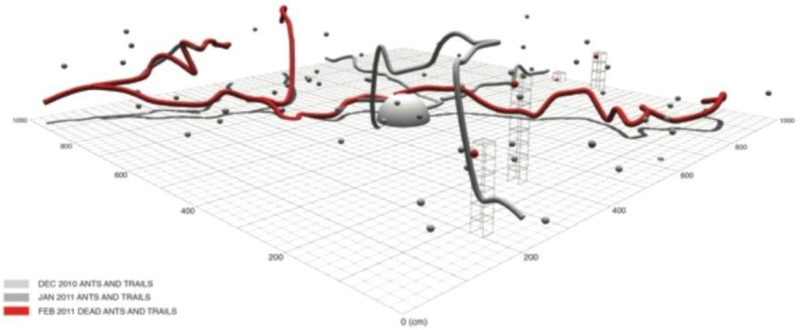
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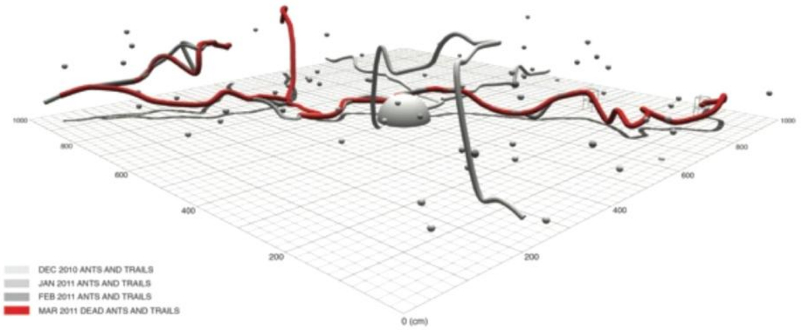
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Figure 2: Distribution of dead *Ophiocordyceps camponoti-rufipedis* infected ants (dots) and foraging trail of host ant *Camponotus rufipes* (lines) across four months around the colony B, in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, southeastern Brazil. (A) December. (B) January. (C) February. (D) March. The red dots are the new dead ants for that month. The red lines are the trails for each month. Where the dots or trails are grey these are ants or trails from the previous month(s). The small squares below the dots (dead ants) show the heights of the ants (each square corresponds to 50cm x 50cm).

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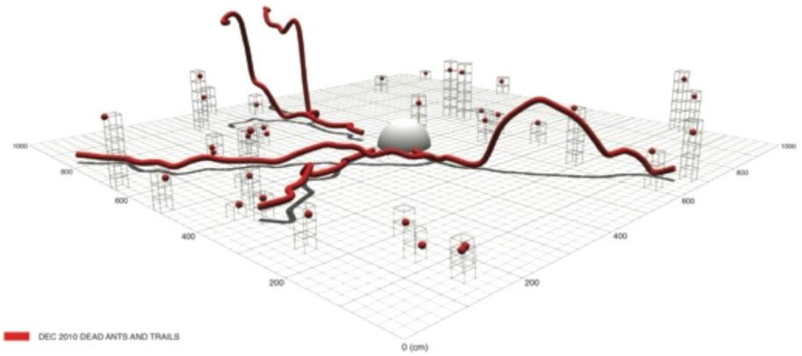

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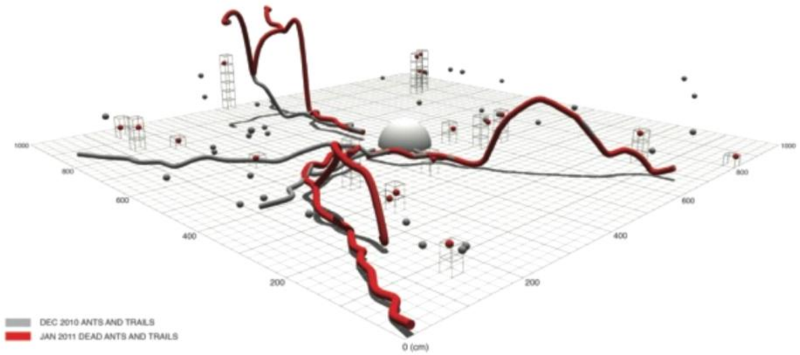

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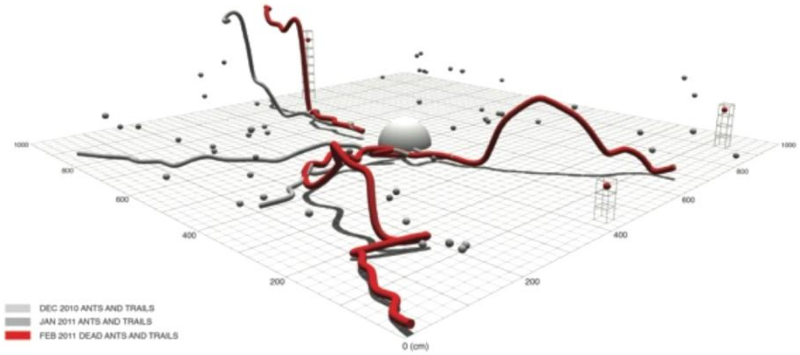

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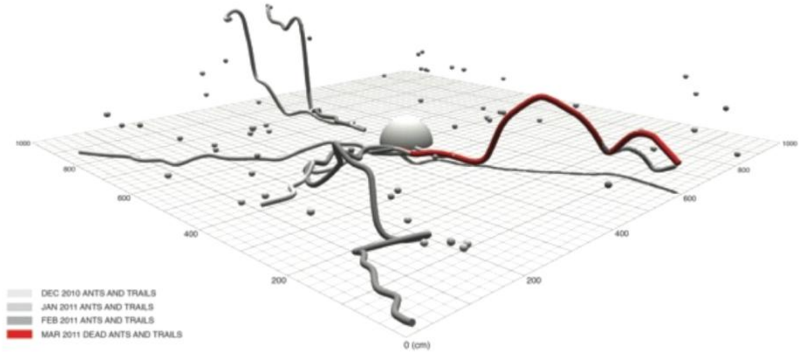

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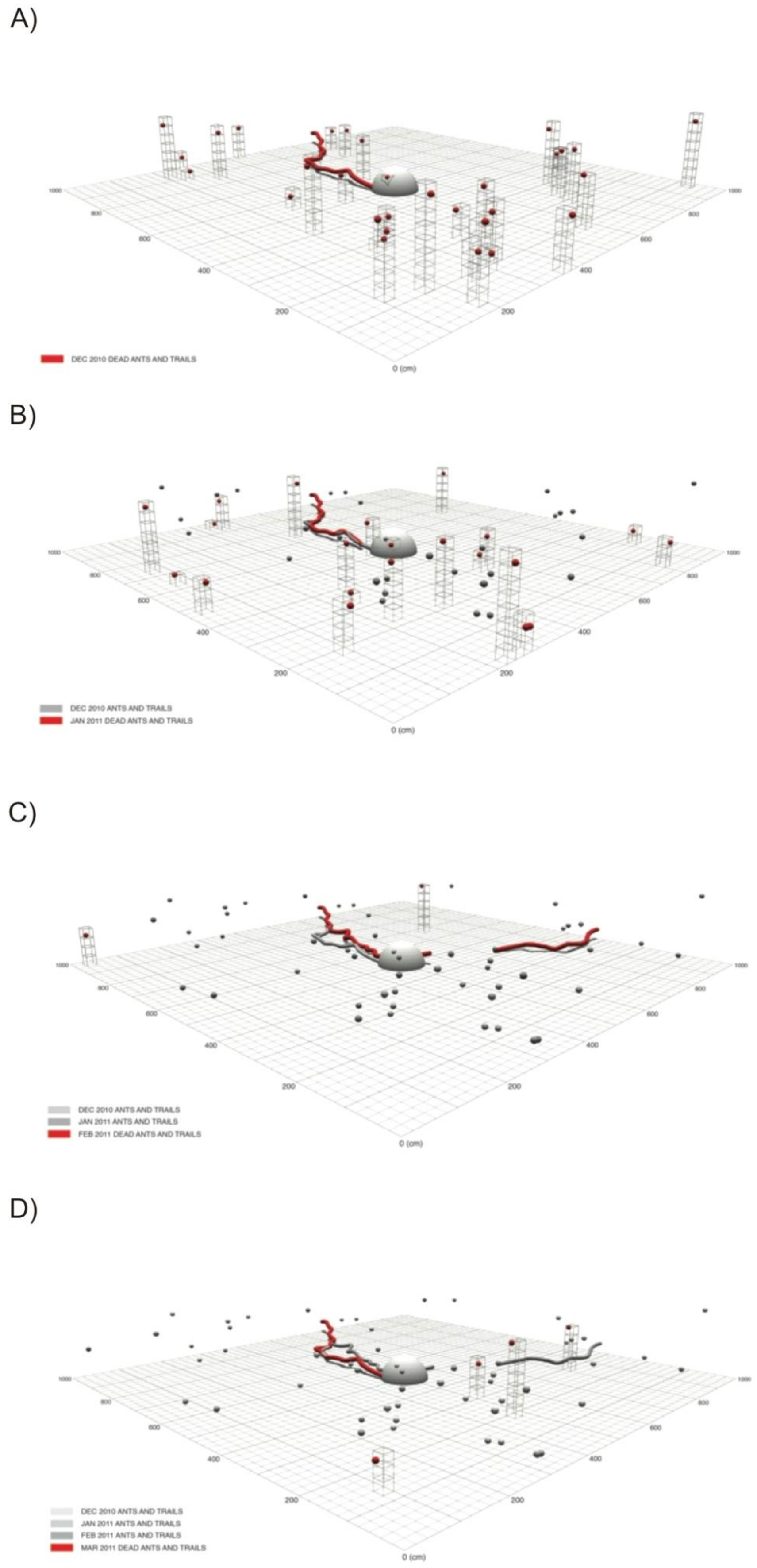
MAR 2011 DEAD ANTS AND TRAILS

Figure 3: Distribution of dead *Ophiocordyceps camponoti-rufipedis* infected ants (dots) and foraging trail of host ant *Camponotus rufipes* (lines) across four months around the colony C, in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, southeastern Brazil. (A) December. (B) January. (C) February. (D) March. The red dots are the new dead ants for that month. The red lines are the trails for each month. Where the dots or trails are grey these are ants or trails from the previous month(s). The small squares below the dots (dead ants) show the heights of the ants (each square corresponds to 50cm x 50cm).


A)
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DEC 2010 DEAD ANTS AND TRAILS
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MAR 2011 DEAD ANTS AND TRAILS

Figure 4: Distribution of dead *Ophiocordyceps camponoti-rufipedis* infected ants (dots) and foraging trail of host ant *Camponotus rufipes* (lines) across four months around the colony D, in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, southeastern Brazil. (A) December. (B) January. (C) February. (D) March. The red dots are the new dead ants for that month. The red lines are the trails for each month. Where the dots or trails are grey these are ants or trails from the previous month(s). The small squares below the dots (dead ants) show the heights of the ants (each square corresponds to 50cm x 50cm).

Although the number of new dead ants was variable between the four plots (F(3,15) = 10.018; P<0.01), the pattern of newly dead ants was similar for all plots throughout the study period (Figure 5).

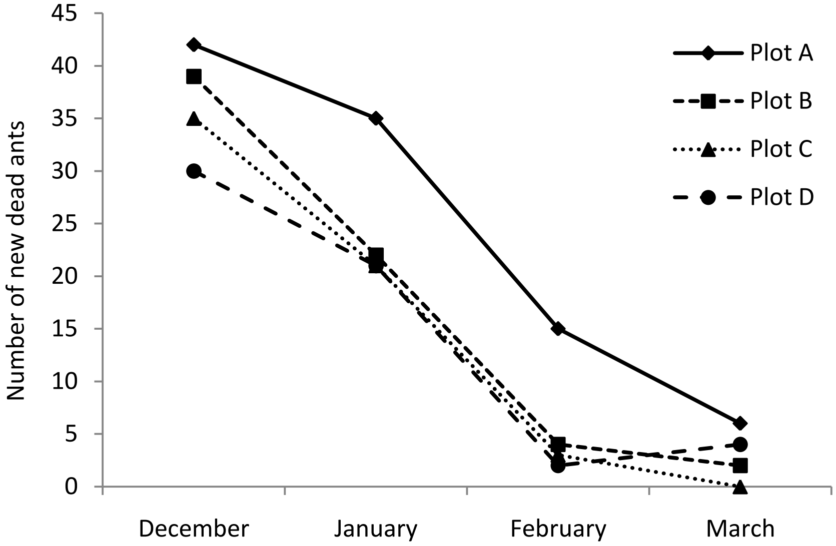

Figure 5: Number of new dead *Ophiocordyceps camponoti-rufipedis* infected ants (*Camponotus rufipes*, Formicinae: Camponotini) in four months (December 2010 to March 2011) in four 100m^2 plots (A,B,C and D) in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, southeastern Brazil. On center of each plot was a *C. rufipes* nest.

The number of new mycozed dead ants was dependent on the month (ANCOVA: F(2,15) = 71.79; P<0.001) and the number of trails (F(1,15) = 24.67; P<0.001) (Figure 6). We found more new cadavers in December (36.5±5.19, mean±SD) (F(2,15) = 0.8101) than in January (24.75±6.84, mean±SD) (F(2,15) = 17.402; P<0.01). In February and March we found the same mean of new dead ants (4.50±4.50, mean±SD) (F(2,15) = 0.8101; P=0.552), which was lower than January (F(2,15) = 68.915; P<0.001). It is known, in general, that fungi are sensitive to environmental conditions (13) and, the parasite *O.*

unilateralis is no different (11). In the study area, the months of November, December and January represent 75% of the year precipitation (14). Thus, the greater numbers of new dead ants in these months is to be expected.

The number of trails varied between colonies and months (Figures 1 to 4), but as seen in Chapter 1, the ants maintained their foraging trail over time, and, after two months, 50% of the trail was unchanged (Chapter 1, Figure 5). We noted some variation in intensity of activity between colonies, measured by the number of new trails across the four months. For colony A, we found a total of eight trails from December to March; for colony B, seven; colony C, four; and colony D, two. As the ants can only get infected outside the nest, the high activity may represent a high number of possible new hosts for the fungus. In fact, we found a relationship between the number of trails and new infected dead ants (ANCOVA: F(1,15) = 24.67; P<0.001) (Figure 6).

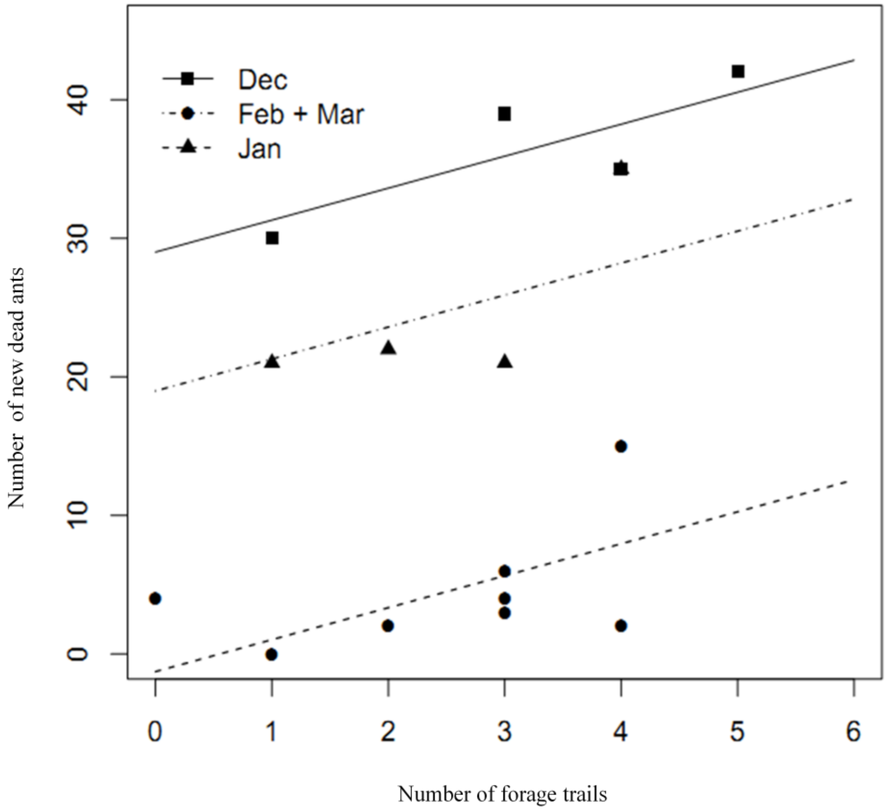

Figure 6: Positive relationship between the number of new dead *Ophiocordyceps unilateralis* infected ants for each month and number of forage trails 100m^2 around four colony of ant host (*Camponotus rufipes*) (Colonies A,B,C and D), in a recovered fragment of Atlantic rainforest, Viçosa, Minas Gerais, southeastern Brazil.

Fungus *versus* ant height. Corroborating the hypothesis of optimized control of the parasite over the host, we found the dead ants to be positioned higher (65.23±21.67 cm (mean±SD)) than the healthy ants (36.74±17.67 cm (mean±SE)) ($\chi^2(3) = 13.65$; $P<0.001$) (Figure 7). This pattern was constant across time, having no differences between the four months ($\chi^2(3) = 0$; $P=1$). It has been suggested that one of the advantages for the ants in using bridges as a part of their trails could be the possibility to walk above the infected dead ants (Chapter 1), which are the source of the parasite's infective stage. This is not the case, however, and even using bridges and walking on aerial trails, the healthy ants are within the fungus' range. In contrast, Pontoppidan et al. (2009) (11) found that the main host of *O. unilateralis* in Thai rainforest, the ant *C. leonardi*, constructs the foraging trail in the canopy, much higher than they found the infected dead ants. As reported recently, each ant is parasitized by a specific fungus species (15) and the fungus parasite of *C. rufipes* may be more able to optimize the manipulation of it host increasing its own fitness.

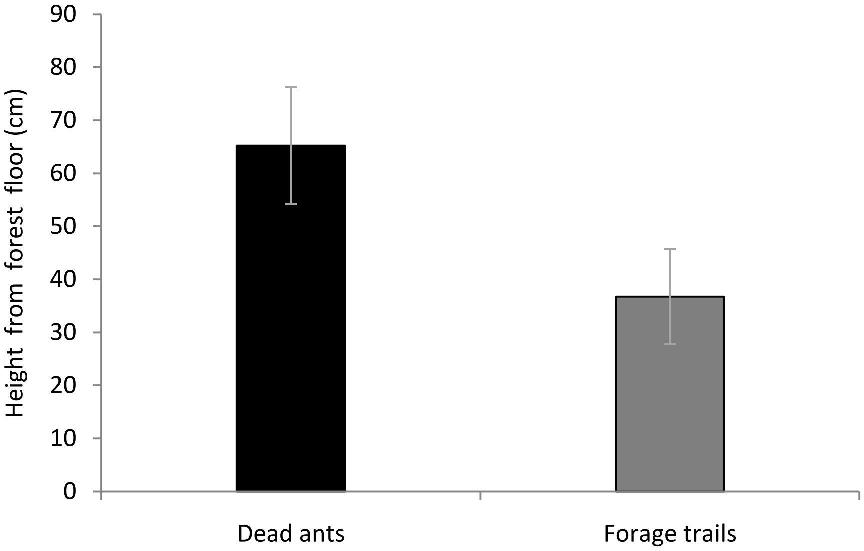

Figure 7: The mean (±SE) height from forest floor (in centimeter) of dead ants infected by the parasitic fungus *Ophiocordyceps unilateralis* (black bar) and of forage trails (gray bar) of the host ant (*Camponotus rufipes*) in four different colonies (Colonies A,B,C and D). The heights (z coordinate – see Material and Methods) were collected across four months (December 2010 to March 2011), once per month.

Spatial distribution of infected dead ants. We calculated the distances of infected dead ants found in December to the nearest ant forage trails and compared these with the distances of randomly generated points in space from the nearest ant forage trails (also found in December). We concluded that the dead ants are randomly distributed with respect to the trails as we found no difference (F(1,245) = 0.0121; P = 0.9125). As the distances were not variable between the four months (F(3,83) = 0.4799; P = 0.6972), we expect this lack of a relation between localization of dead ants and of healthy ants to persist in the remaining months. If the control of fungus over host were total, we might expect to find infected ants dying close to the trail, but they did not.

Although we did not find a relation between localization of the trail and of infected dead ants, it is possible that the host forage trails are nevertheless within the fungus' dispersal range. Spores of *O. unilateralis* are too large to be dispersed by wind, so they should disperse directly to the ground (15). We have shown the dead ants are positioned higher than ants forage trail, so the spores that are shot off may cover the trails. The *O. unilateralis* stroma has a semispherical ascoma, from where the spores are shot (16). When it releases the spores, they may cover a ratio area below the infected dead ant. This range should be unique for each ant cadaver, depending on its height.

As we do not know the ratio range of the spore below the dead ants (we are working to identify this), we used three different hypothetical ranges (height of dead ant/2; height of dead ant/1; and height of dead ant /0.067) to simulate the covering area of each ants we found in December (Figure 8). With the simulation, we can see that even if the spore converge in a small area below the ant, represented by half its height (Figure 8A), it would still cover part of the ants' foraging trail. An intermediate hypothetical ratio range (Figure 8B) shows almost the entire trail length could be hit by the fungus and with a high hypothetical ratio range (Figure 8C) shows the plot would be almost completely covered by spores. We recognized the ratio range cover of spore should be calculated, but by our simulation we hypothesized that, even randomly distributed, the dead infected ants cover, at least, part of the ant trails.

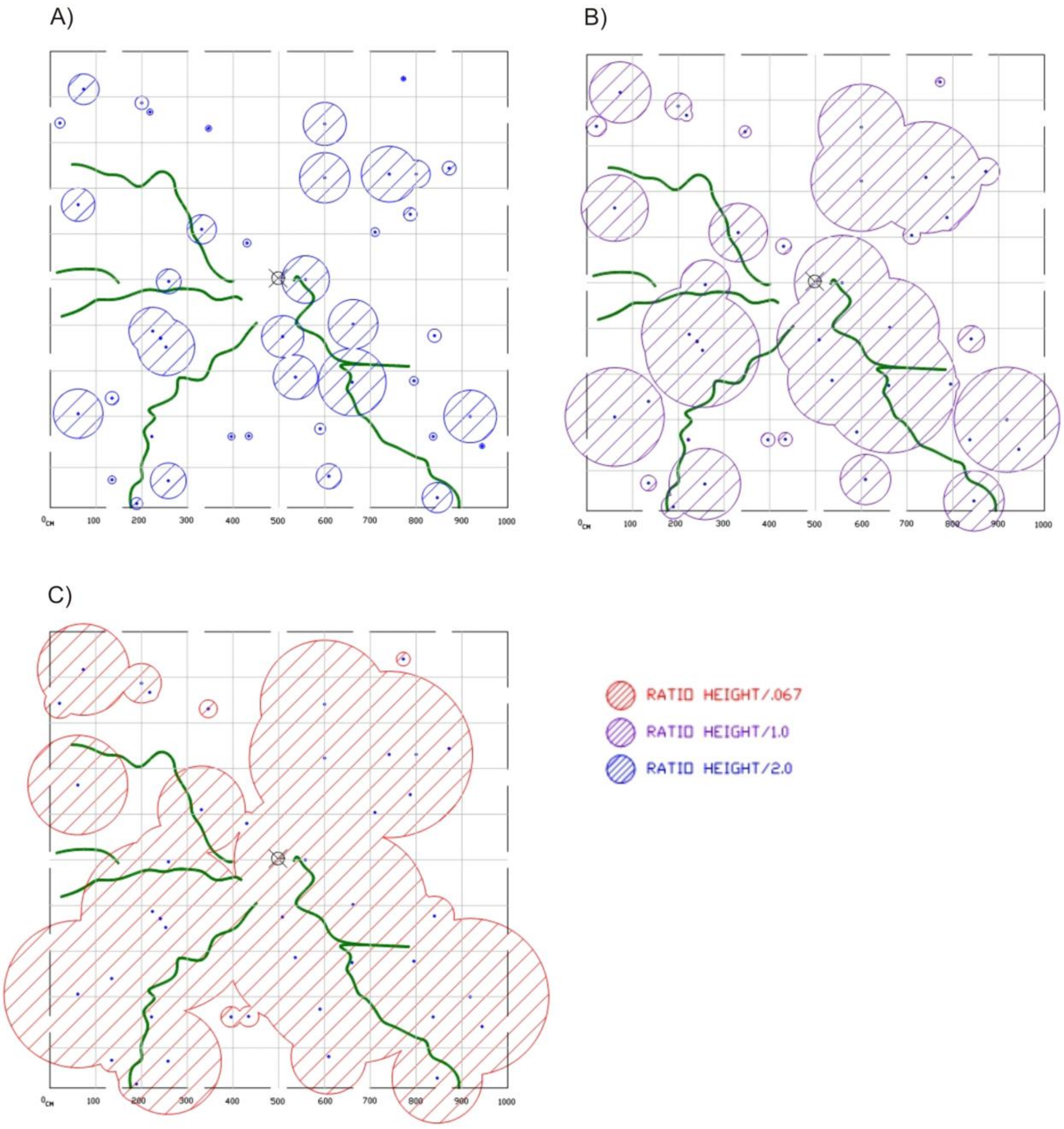

Figure 8: Hypothetical spore range of *Ophiocordyceps camponoti-rufipedis* simulated according to infected dead host height, the ant *Camponotus rufipes*, and special distribution of forage trail. The bubbles are the spore covering area and green lines are the ant trail. (A) Hypothetical covering spore area for spore small range equivalent to dead ant height/2. (B) Hypothetical covering spore area for intermediated spore range equivalent to dead ant height. (C) Hypothetical covering spore area for spore high range equivalent to dead ant height/0.067.

Parasitic manipulation can result in dramatic behavioral changes, such as spiders constructing new styles of web (17) or crickets committing suicide in water (6), or it can result in more moderate changes, such as changes in microhabitat (18). The fungus *O.*

unilateralis is sensitive to microclimate conditions, and it manipulates the host to die in a specific place to optimize its subsequent growth (11). However, we would expect hosts to fight infection (19) or at least ameliorate its costs. It is known that the ant host *C. rufipes* constructs its forage trail mainly using bridges, objects found in the forest (fallen branches, lianas and twigs), increasing its overall speed. It has been suggested that this also serves as a strategy to avoid fungal spores (chap 1). Using the bridges, the ants visibly decrease their contact area with the environment (we are working to quantify this) and, even within the spore range that covers the ant trail, a small area of this spore range is thus covered by ants. This may be a strategy to decrease the infection rate, especially at the colony level (20). Thus, even if the trails are within the fungus' range, this may not represent a great risk for the ants since their trails are maintained through time (chapter 1).

After a host gets infected, it may alter its behavior to minimize the consequences of infection (19). Considering the host studied here is a social insect and the parasite is an obligate killer, the ant may increase its (inclusive) fitness by protecting the colony from the parasite. The random localization of infected dead ants related with the trail, is consistent with the theory of social isolation, which was tested with workers of the ant *Temnothorax unifasciatus*: they leave their group to die in social isolation (10).

We expect that some ants are able to recover from infection, as can leaf-cutter ants (21), through their immune system (19). Such recovering ants would not have been sampled in our study, being considered healthy ants. In addition, the ants also could fight against the fungal control and die in other locations, for example, on the ground, where the fungus does not grow (11). In this case, they would disappear in less than 24h (11). Previous work on the zombie ant system documented a precise and fine detailed level of control with infected workers dying on distinct parts of the leaves, on leaves at a particular height off the ground and orientated in a distinct angle in space (11). Further, the timing of the manipulation was synchronized around solar noon and involved destruction of ant mandibular muscles (22). With our results, we suggest that the fungus may not be in total control of zombie ant, as has been speculated (10,11,22).

Material and Methods

Study area and ant specie. Fieldwork was carried out from December 2010 to April 2011 at the Research Station of Mata do Paraíso, Viçosa, Minas Gerais, Southeast Brazil (20°48'08 S 42°51'31 W). This is a recovered fragment of Atlantic rainforest (it was previously a coffee plantation) where the dominant vegetation is a secondary seasonal semi-deciduous montane forest (23). Although it is characterized by a robust understory, foliage is not too thick at ground level. The ground is typically covered with 10-20cm of leaf litter, though there are patches where soil is exposed. *Camponotus rufipes* is very abundant in this fragment, feeding on nectar and honeydew and on live arthropods (Jaffe & Sanchez 1984, Del-Claro & Oliveira 2000). In natural habitat, colonies of *C. rufipes* are active at night, with a maximum activity in early night. Their foraging trails are mainly builder over bridges (fallen branches, lianas and twigs) and it makes the ant use the 3D space on forest, not walking only on the floor (Chapter 1).

Data collection. *Healthy ants.* Four established nests of *C. rufipes* were identified. These were approximately 1-2m in diameter and were built on the forest floor, up against live trees. A plot (10m x 10m) was demarcated around each nest, such that the nest entrance was central. Each plot was sub-divided into a 25-square grid (2m x 2m) and foraging trails were marked with small flags placed every 30cm, starting at the nest and continuing until they left the plot. This was done shortly after dusk, when foraging peaked. Different colored flags were used to identify those parts of the trail following bridges (fallen branches, twigs and lianas) and those occurring on the forest floor. The coordinates of each flag inside the plot were determined by measuring the x, y and z position. The z positions were measured from the forest floor. The trails were flagged and monitored in this manner once a month for four months (December to March). These data are from Chapter 1.

Dead ants. We checked every single leaf, until 1.60 meter, inside the four plots cited above in order to find the dead ants by the *Ophiocordyceps unilateralis.* Each dead ant found was marked with a tag, in which was written the identification number of the ant and the date. The first survey (November 2010) was done in order to identify all ants there died before we start the study. The data collecting was done in December 2010, January, February and March 2011 (and will continue in May and June, but these new data will be part of the final paper). The relative position of new dead ants inside the plot was measured with x, y and z coordinates, as the healthy ant position. Each four weeks, we did one survey, looking for new dead ants. All analyzes were conducted using the free software R (version 2.12.0).

System description. As the system has never been studied before, we described the dynamic of infection by *O. unilateralis* and the dynamic of host's trails across four months. We conducted an ANOVA to compare the number of ants killed by the fungus. The data were plotted as 3D images using the formZ software (AutoDesSys – www.formz.com).

Fungus *versus* ant height. In the context of the perfect control hypothesis, we should expect the ant trails to be lower than the dead ants; since the ants use bridges to make the trail (Chapter 1). To test this, we used the z coordinate, collected as described above, which represents the height of the two different populations: the fungus and the host. The two groups of data were contrasted in an ANOVA, with mixed models to eliminate temporal pseudo-replications.

Spatial distribution of dead ants. We hypothesized that if the fungus control over ant is complete, the infected ants should die close to the trail, where the healthy host is. To test this hypothesis, we aim to contrast the distance of each dead ant and random points on space until the foraging trail. We calculated the distance of each dead ant found in December to the nearest trail (also found in December), using the x, y and z coordinates in the software Grasshopper® (www.grasshopper3d.com). We also asked the software for 204 random coordinates which represent random points on space. For each of these random coordinates we calculated the distance from the nearest trail present in December. The two groups of distances were contrasted. The distance was used as the response variable group of distance (dead ants or random points) as explanatory variable, while each ant was used as a replicate. We also aim to know if the distance of ants from trails were constant across the four months. To test this, we conducted a further ANOVA using only the distance of real ants as the response variable and the months (December, January, February and March) as explanatory variables.

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CONCLUSÃO GERAL

Nós concluímos que a formiga carpinteira *Camponotus rufipes* otimiza suas trilhas de forrageamento usando caminhos mais longos e rápidos, e não caminhos mais curtos porém mais lentos. Essa é uma nova visão de otimização de forrageamento de formigas, conceito que tem sido usado para inspirar solução de problemas em ciências computacionais. Nós também geramos novas informações sobre o forrageamento de um importante grupo de formigas, mostrando que elas usam objetos previamente dispostos na floresta (pontes) para construir suas trilhas de forrageamento. Essas trilhas são persistentes ao longo do tempo, caracterizando trilhas tronco.