Imidacloprid Inhibits Behavioral Defences of the Leaf-Cutting Ant *Acromyrmex subterraneus subterraneus* (Hymenoptera:Formicidae)

Jefferson P. Galvanho · Marinete P. Carrera ·
Denise D. O. Moreira · Milton Erthal Jr. ·
Carlos P. Silva · Richard Ian Samuels

Abstract Leaf-cutting ants have evolved a range of defensive strategies which complicate the use of entomopathogens for their control. One of these behavioral strategies is self-grooming, which increases when ants detect the presence of fungal conidia on their integuments. We have previously shown that insecticides, when used at ultra-low concentrations, can be synergists of entomopathogenic fungi. It is possible that certain insecticides could modify ant behavior in a way that increases the chances of a fungal infection taking hold. The current study investigated the effects of the neonicotinoid insecticide Imidacloprid (IMI) and the entomopathogenic fungus *Beauveria bassiana* on self-grooming and locomotion behavior of *Acromyrmex subterraneus subterraneus* workers when ants were exposed to the agents separately or together. Initially 10, 20 and 40 ng/insect IMI was topically administered to worker ants. Four hours after administration, the ants were placed into an experimental arena and self-grooming and locomotion behaviors were recorded for 10 min. Separate groups of ants were exposed to filter paper discs impregnated with

J. P. Galvanho · D. D. O. Moreira · R. I. Samuels
Department of Entomology and Plant Pathology, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ 28013-602, Brazil

M. P. Carrera
Behavioral Pharmacology Group, Laboratory of Morphology and Pathology Animal, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ 28013-602, Brazil

M. Erthal Jr.
Universidade Candido Mendes-Campos, UCAM-CAMPOS, Candido Mendes, Brazil

C. P. Silva
Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, SC, Brazil

R. I. Samuels (✉)
UENF-CCTA-LPP, Av. Alberto Lamego 2000, Campos dos Goytacazes, RJ 28013-622, Brazil
e-mail: richard@uenf.br

conidial suspensions and 4 h later their behavior was monitored for 10 min. Subsequently, ants were treated with 10 ng/insect IMI, immediately exposed to fungal suspensions and 4 h later their behavior was monitored. The 10 ng/insect IMI treatment resulted in higher locomotor activity and lower self-grooming when compared to vehicle, 20 ng and 40 ng IMI treatments. Exposure to fungal conidia produced low locomotion and high self-grooming behaviors. The 10 ng IMI + fungal conidia treatment resulted in lower self-grooming and higher locomotion behavior. These alterations caused by a low concentration of IMI could increase ant susceptibility to infection by entomopathogenic fungi and are thus of interest for future tests in the development of integrated pest management strategies.

Introduction

Leaf-cutting ants are serious pests in the Neo-tropics and notoriously difficult to control (Della Lucia 2011). Currently the only control methods available are based on the use of insecticides (Della Lucia and Vilela, 1993). Alternatives such as biological control using entomopathogens have been tested in the field without success (Della Lucia 2011). The highly advanced level of social organization seen in leaf-cutting ants of the Attini tribe (*Atta* and *Acromyrmex*) could be responsible for the failure of entomopathogens to cause epizootics within colonies. The social organization includes behavioral defensive strategies such as self-grooming, allo-grooming, fungus garden grooming/weeding and exclusion of unhealthy colony members (Currie and Stuart 2001). Apart from behavioral strategies, the ants posses an array of chemical defences which include the secretion of antibiotic compounds from the metapleural glands (Bot et al. 2002), ingestion of contaminating material which is rendered innocuous by enzyme and antibiotic secretions in the infrabucal pocket (Little et al. 2006), antibiotics produced by symbiotic actinobacteria associated with the integument (Currie et al. 1999; Mattoso et al. 2011) and antimicrobial secretions from bacteria found in the fungus garden (Santos et al. 2004). Therefore it is important to increase our knowledge of leaf-cutting ant defensive barriers in order to develop more efficient forms of pest management. It is possible that combining insecticides with entomopathogenic fungi could be used to break the behavioral defensive barriers of social insects, thus allowing fungi to kill significant numbers of colony members, resulting in colony collapse.

Santos et al. (2007) demonstrated synergistic effects of low concentrations of the neonicotinoid insecticide Imidacloprid (IMI) on the virulence of the entomopathogenic fungus *Beauveria bassiana* when used together against the leaf-cutting ant *Atta sexdens*. It is possible that this insecticide modifies grooming behavior in leaf-cutting ants as IMI has been observed to interfere with the social behaviors of termites such as trophallaxis, grooming and tunnel construction (Boucias et al. 1996).

Interestingly, there are very few reports of entomopathogenic fungi naturally attacking leaf-cutting ants (Hughes et al. 2009), whilst other ant genera such as *Camponotus* suffer from regular epizootics in tropical rainforests (RIS personal observation). Although laboratory studies have shown the susceptibility of leaf-

cutting ants to entomopathogenic fungi (Santos et al. 2007), little success has been seen in the field unless very high concentrations of fungal conidia are applied directly to the nests (Diehl-Fleig et al. 1993).

One of the major defensive behaviors utilized by leaf-cutting ants to protect themselves and their fungus garden from parasites is self-grooming and allo-grooming. Wilson and Regnier (1971) described the grooming process as an activity involving the use of the tibial combs to clean the body surface followed by licking of the tarsi. In this way the ants can remove spores of contaminating fungi from their integuments, which are subsequently compacted into pellets in the infrabucal chamber, where they are exposed to chitinolytic secretions (Febvay and Kermarrec, 1981) or antifungal compounds (Little et al. 2006), inactivating the spores. The pellets are then discarded in the trash sector of the nest (Oi and Pereira, 1993; Little et al. 2006).

Neuro-toxic insecticides are known to alter behavior. An interesting example is Imidacloprid (IMI), a synthetic chemical insecticide widely used for controlling insect pests, although not registered for use against leaf-cutting ants. Its mechanism of action is related to its nicotinic acetylcholine receptor affinity (Elbert et al. 1991) and has thus been classified as a neonicotinoid (Matsuda et al. 2001). Other nicotinic agonists such as nicotine itself are capable of producing behavioral changes in insects, exacerbating locomotion at low doses and decreasing locomotion at high doses (Bainton et al. 2001). According to Lambin et al. (2001), IMI produces dose dependent changes in locomotor behavior in *Apis mellifera.*

We tested the effects of IMI on self-grooming and locomotion with the aim of modifying the behaviour of the leaf-cutting ant *A. subterraneus subterraneus* in order to increase susceptibility to fungal infection. A reduction in self-grooming would be predicted to increase the rate of infection of fungus exposed ants. Following establishment of a behavioral modification protocol which resulted in a reduced level of self-grooming, we then exposed ants to both IMI and fungal conidia simultaneously with the aim of reducing one of the defensive strategies employed by leaf-cutting ants against entomopathogen infection.

Methods

Insects

Worker ants of *A. subterraneus subterraneus* were obtained from three individual colonies maintained in laboratory (Della Lucia 1993) at 25°C and a photoperiod of 12 L: 12D. Only worker ants with head capsule widths of 1.6–2 mm, denominated as C3 by Moreira et al. (2010), were utilized in the experiments. All experimental groups consisted of an equal number of individuals from each of the three colonies. The ants were maintained solely on a diet of *Acalifa sp.* leaves. Behavioral tests were always carried out during the light phase from 13:00 h to 17:00 h.

Insecticide

The commercially available insecticide Confidor® (70 % Imidacloprid and 30 % inert substances supplied by Bayer Brazil) was used in all experiments at concentrations of

10, 20 and 40 ng/insect. IMI was diluted in sterile distilled water and administered topically using a Gilson micropipette onto the dorsal thorax in a volume of 1 μl. A sterile distilled water solution was used as vehicle for the IMI experiments. Solutions were freshly prepared before each experiment.

Fungal Isolates and Preparation of Suspensions

An isolate of *Beauveria bassiana* from the collection at CENARGEN (Centro Nacional de Recursos Genéticos) denominated CG24, was used in all experiments here as it had previously been demonstrated to have high virulence against leaf-cutting ants of the genus *Atta* and *Acromyrmex* (Santos et al. 2007; RIS: unpublished data). Fungi were cultured on Sabouraud Dextrose Agar (Dextrose 10 g; Peptone 2.5 g; Yeast Extract 2.5 g; Agar 20 g in 1 L H_2O) at 27°C for 15 days before being used in experiments. Fungal suspensions were initially prepared in Tween 80 (0.05 % in sterile distilled water) and conidial concentration determined using a Neubauer hemocytometer. A final concentration of 1×10^8 conidia mL^{-1} was prepared by serial dilution. Fungal suspensions were vortex mixed vigorously before applying 1 mL to filter paper discs (6 cm diameter) using a pipette. Tween 80 (0.05 % in sterile distilled water) solution was used as vehicle for the experiments with fungi.

Apparatus and Measurement of Behaviour

All tests were carried out in a controlled environment at a temperature of 25±2°C and 80 % relative humidity. The test arena consisted of a plastic Petri dish (10 cm diameter and 1.5 cm height). A closed-circuit camera (SONY, model IR575M), mounted 60 cm above the arena was used to record behavioural data. Locomotion, measured as distance traveled (m), was automatically analyzed using EthoVision software (Noldus, The Netherlands). Self-grooming was quantified as the time (in seconds) that the ants carried out the following behaviors: passing one leg over the other, rubbing the forelegs with the mouthparts, antennae with the mouthparts or front legs, the abdomen with the mouthparts or with the hind legs and head with the tarsi. Self-grooming was analyzed by a trained observer who was unaware of the treatment under test.

Experimental Procedure

Initially, the ants were removed from the foraging area of their nests with forceps, placed individually in Petri dishes and taken to the experimental room where they were left to acclimatize for a period of 1 h. All the experimental groups consisted of an equal number of individuals from each of the nests. After the acclimatization period and in order to verify the effect of three different concentrations of IMI on locomotion and self-grooming behavior (experiment 1), separate groups of ants received vehicle (VEH), 10 ng IMI (IMI-10), 20 ng IMI (IMI-20) or 40 ng IMI (IMI-40) and were left for 4 h in the acclimatization Petri dishes. After that period, the ants were individually placed into the experimental arena and locomotion and self-grooming were monitored for 10 min. Subsequently, in order to verify the effect of exposure of ants to fungal conidia on self-grooming behavior and locomotion

(experiment 2), two separate groups of ants were exposed to either vehicle (VEH) or fungal conidia (FUNGUS) for 4 h. After that period, the ants were individually placed into the experimental arena and locomotion and self-grooming were monitored for 10 min. In order to verify the effects of co-administration of IMI + fungus on self-grooming and locomotion (experiment 3), two separate groups of ants received vehicle (VEH) or 10 ng IMI and were exposed to fungal conidia (IMI-10+F) for 4 h before locomotion and self-grooming were measured for 10 min. Each experimental group consisted of 24 ants.

Statistics

In order to carry out within treatment assessments of the behavioral activity data, the total test time (10 min) was divided into 2 intervals of 5 min duration each and a repeated two-way ANOVA consisting of between-subject factor group and a repeated-measurements factor interval was used. Wherever indicated by the ANOVA (group effects with p-values<0.05), possible differences among groups were analyzed by Duncan's test or by an independent t-test.

Results

In order to verify that there were no differences in the behaviors evaluated (locomotion and self-grooming) between the three different colonies; a one-way analysis of variance (factor colony) was performed. The test showed no significant differences between the three colonies, for both self-grooming [$F_{(2, 237)}=0.03$; $p>0.5$] and locomotion [$F_{(2, 237)}=0.39$; $p>0.5$] (data not shown).

Figure 1 shows the dose–response curves of self-grooming and locomotion behavior following application of three concentrations of IMI. For self-grooming (Fig. 1A), a repeated two-way ANOVA indicated that there was an effect of treatment [$F_{(3, 92)}=5.22$; $p<0.01$], an effect of interval [$F_{(1, 92)}=26.0$; $p<0.01$] but no interaction treatment X interval [$F_{(3, 92)}=0.66$; $p>0.05$]. According to Duncan's test, the IMI-10 treatment exhibited lower levels of self-grooming behavior when compared to the other treatments ($p<0.05$). There were no differences in self-grooming behavior between VEH, IMI-20 and IMI-40 ($p>0.05$). For locomotion (Fig. 1B), a repeated two-way ANOVA showed that there was an effect of treatment [$F_{(3, 92)}=10.50$, $p<0.01$], an effect of interval [$F_{(1, 92)}=11.08$; $p<0.01$] but no interaction treatment X interval [$F_{(3, 92)}=0.42$; $p>0.05$]. According to Duncan's test, the results show that IMI-10 had higher locomotor activity than all other treatments ($p<0.05$).

Figure 2 shows the effect of exposure of ants to fungal conidia on self-grooming behavior and locomotion. For self-grooming (Fig. 2A), a repeated two-way ANOVA indicated that there was an interaction treatment X interval [$F_{(1, 46)}=13.0$; $p<0.01$], an effect of treatment [$F_{(1, 46)}=83.71$; $p<0.01$] and an effect of interval [$F_{(1, 46)}=31.0$; $p<0.01$]. A independent t-test to further analyze the interaction treatment X interval, performed for treatments during each interval, showed that at first interval [t (46)=7.20; $p<0.01$] and second interval [t (46)=10.40; $p<0.01$], the fungus treated group spent more time self-grooming than the ants of the vehicle group ($p<0.01$). The results also showed (paired t-test) that self-grooming times decreased across the

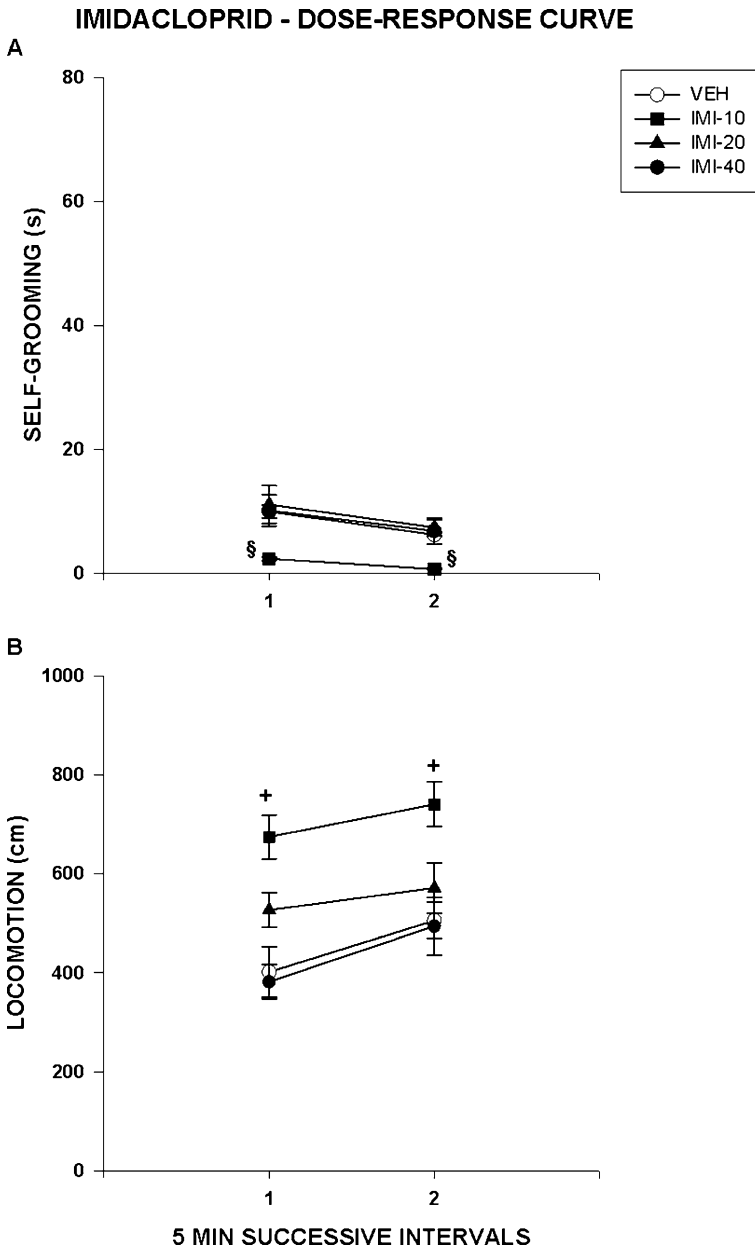## IMIDACLOPRID - DOSE-RESPONSE CURVE

Fig. 1 Means and S. E. M. of effects of administration of imidacloprid (IMI) 10, 20 and 40 ng/insect on self-grooming **A** and locomotion **B**. + denotes significantly higher behavioral activity than the other groups. § denotes lower behavioral activity than the other groups ($p<0.05$; ANOVA followed by Duncan's multiple range test). $N=24$ ants for each group

interval for the vehicle group [t $(23)=5.50$; $p<0.01$] and for the fungus treated group [t $(23)=4.60$; $p<0.01$]. For locomotion (Fig. 2B), a repeated two-way ANOVA showed that there was an effect of treatment [F $_{(1, 46)}=5.40$, $p<0.05$], an effect of
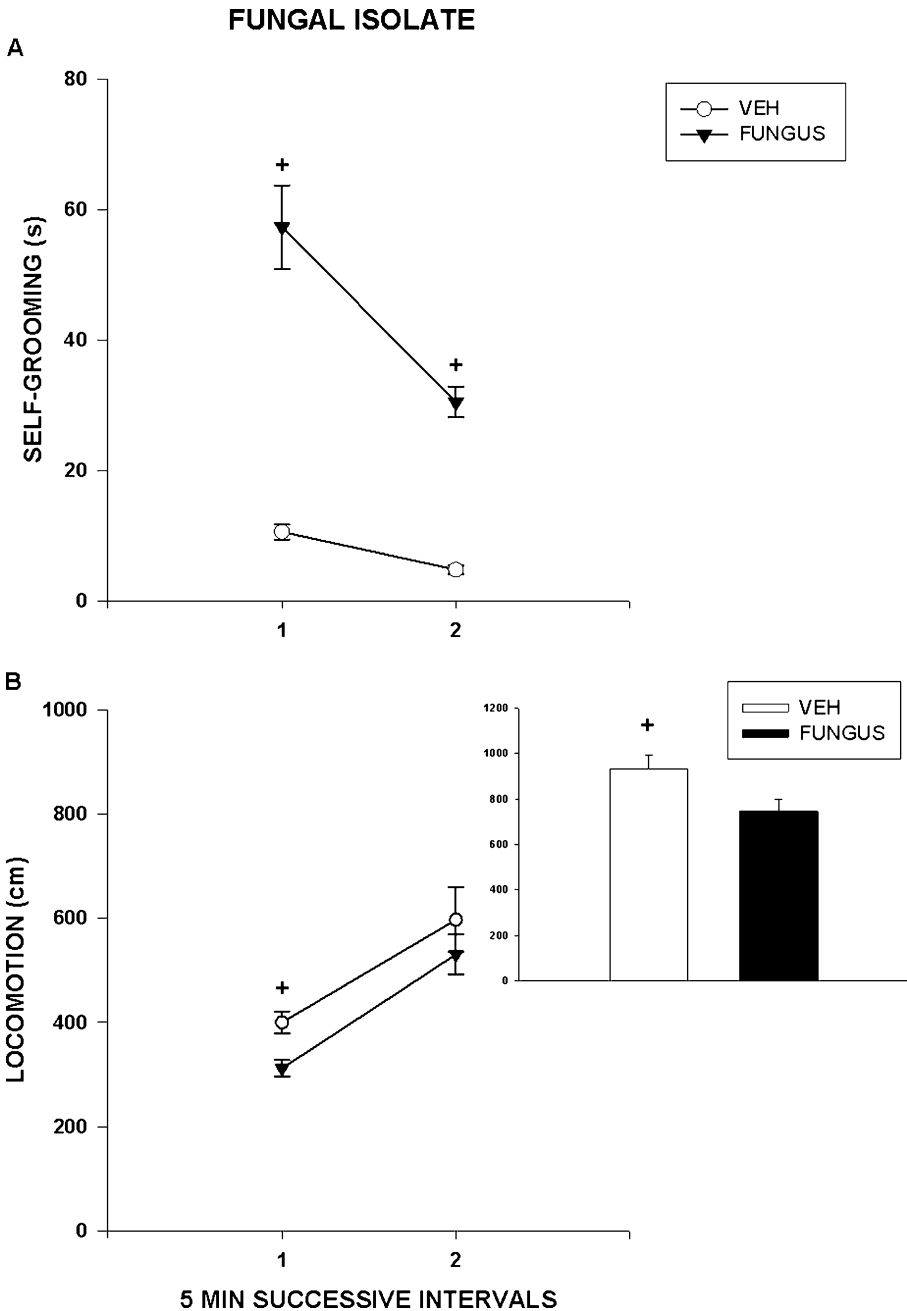
Fig. 2 Means and S. E. M. of effects of exposure of ants to fungal conidia on self-grooming **A** and locomotion **B**. + denotes significantly higher behavioral activity than the other groups. The insert on **B** presents the total 10 min locomotion data on a different scale ($p<0.05$; ANOVA). $N=24$ ants for each group

interval [$F_{(1, 46)}$ 22.50; $p<.01$] but no treatment X interval interaction [$F_{(1, 46)}=0.04$, $p>0.05$]. The results show that the fungus treated insects had lower locomotor activity than the vehicle group ($p<0.05$).

Figure 3 shows the effects of administration of IMI+ exposure to fungal conidia on self-grooming and locomotion. For self-grooming (Fig. 3A), a repeated two-way ANOVA indicated that there was an interaction treatment X interval [$F_{(1, 46)}=30.8$; $p<0.01$], an effect of treatment [$F_{(1, 46)}=44.41$; $p<0.01$] and an effect of interval [$F_{(1, 46)}=104.63$; $p<0.01$]. To further analyze the interaction treatment X interval, an independent t-test was performed between the treatments for each interval and the results showed that for both intervals the vehicle treated group showed higher levels of self-grooming behavior than the IMI-10+F group ($p<0.01$). The results also showed (paired t-test) that self-grooming time decreased across the intervals for the vehicle group [t (23)=8.00; $p<0.01$] and for the IMI-10+F group [t (23)=4.55; $p<0.01$]. For locomotion (Fig. 3B), a repeated two-way ANOVA showed that there was an effect of treatment [$F_{(1, 46)}=11.66$, $p<0.01$], an effect of interval [$F_{(1, 46)}=12.33$; $p<0.01$] but no interaction treatment X interval [$F_{(1, 46)}=0.76$, $p>0.05$]. The results showed that the IMI-10+F treatment showed higher locomotor activity than the vehicle group ($p<0.01$).

In order to verify if co-exposure of ants to IMI-10 and fungal conidia caused a decrease in self-grooming times without interfering with locomotion, a comparison was performed among the IMI-10, fungal isolate and IMI-10+F treatment groups. Initially, an analysis among the 3 vehicle groups was performed and the results showed that there was no statistical difference when considering both grooming behavior [$F_{(2, 71)}=0.26$, $p>0.01$] and locomotion [$F_{(2, 71)}=0.50$, $p>0.01$]. Thus, the vehicle groups were pooled. For self-grooming (Fig. 4A), a repeated two-way ANOVA showed that there was an interaction treatment X interval [$F_{(3, 140)}=25.23$, $p<0.01$], an effect of treatment [$F_{(3, 140)}=152.60$, $p<0.01$] and an effect of interval [$F_{(1, 140)}=65.0$; $p<0.01$]. To further analyze the interaction treatment X interval, a one-way ANOVA followed by Duncan's test was performed for each interval. The results showed that for both intervals, the fungus treated group had higher self-grooming behavior than the other treatments ($p<0.05$). The results also showed that the IMI-10 and IMI-10+F treatments had lower self-grooming times than the vehicle group ($p<0.05$). Self-grooming decreased across the intervals for the fungus treated group ($p<0.05$). For locomotion (Fig. 4B), showed that there was an effect of treatment [$F_{(3, 140)}=22.74$, $p<0.01$], an effect of interval [$F_{(1, 140)}=30.70$; $p<0.01$] but no interaction group X interval [$F_{(3, 140)}=0.40$, $p>0.05$]. The Duncan's test showed that the IMI-10 treatment had higher locomotor activity than the vehicle, fungus treated and IMI-10+F groups ($p<0.05$). The IMI-10+F group had higher locomotion than all other groups ($p<0.05$), except the IMI-10 group.

Discussion

Self-grooming behavior is a very important strategy that has evolved to reduce the risk of infection by parasites. This is important on an individual basis but is even more so when considering social animals, given the high risk of disease transmission between colony members.

Leaf-cutting ant colonies comprise thousands of individuals living in close proximity. Thus pro-active and retro-active disease prevention strategies of these insects are highly evolved. Leaf-cutting ants perform both self-grooming and allo-grooming

to eliminate parasites of the ants themselves and of their fungal gardens. *A. sub-terraneus subterraneus* workers when exposed to conidia of the entomopathogenic fungus *B. bassiana* displayed significantly higher rates of self-grooming than control ants. It has been observed that *Acromyrmex echinatior* workers perform self-grooming before entering nest chambers which contained fungus gardens or brood
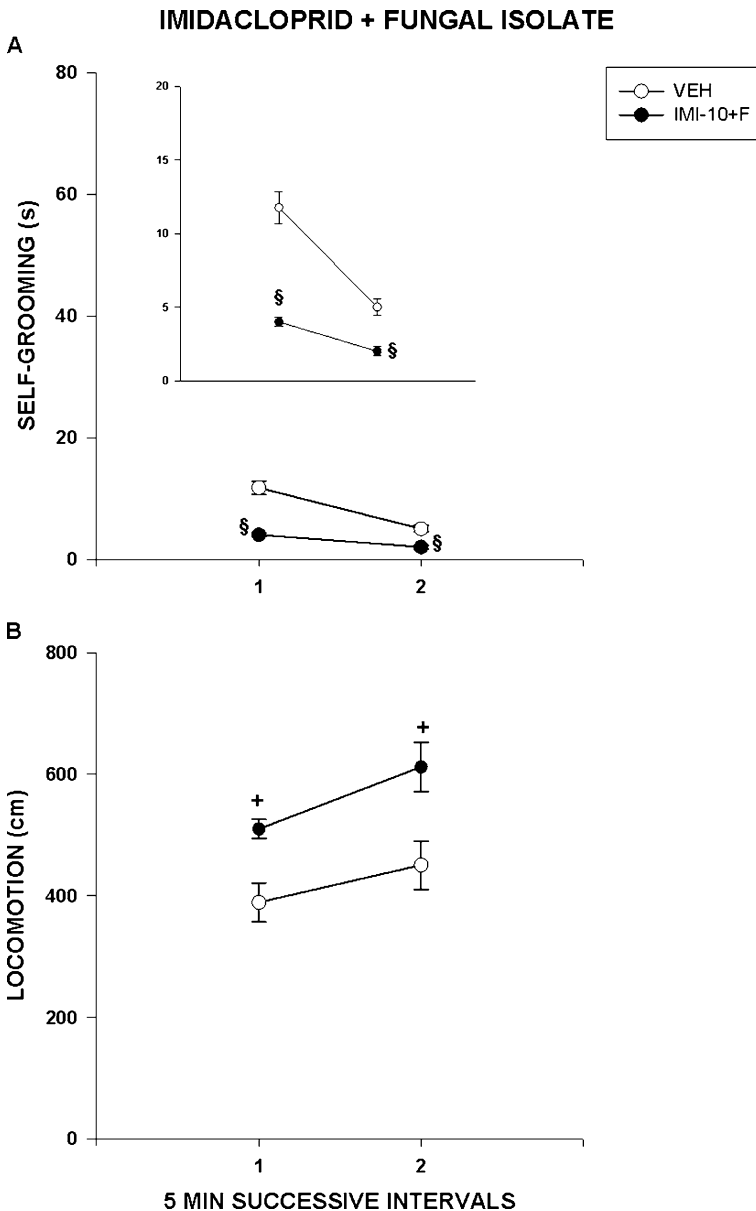

Fig. 3 Means and S. E. M. of effects of administration of IMI 10 ng/insect and exposure to fungal conidia on self-grooming **A** and locomotion **B**. + denotes significantly higher behavioral activity than the other groups. The insert on **A** presents the same self-grooming data on a different scale. § denotes lower behavioral activity than the other groups ($p<0.05$; ANOVA). $N=24$ ants for each group
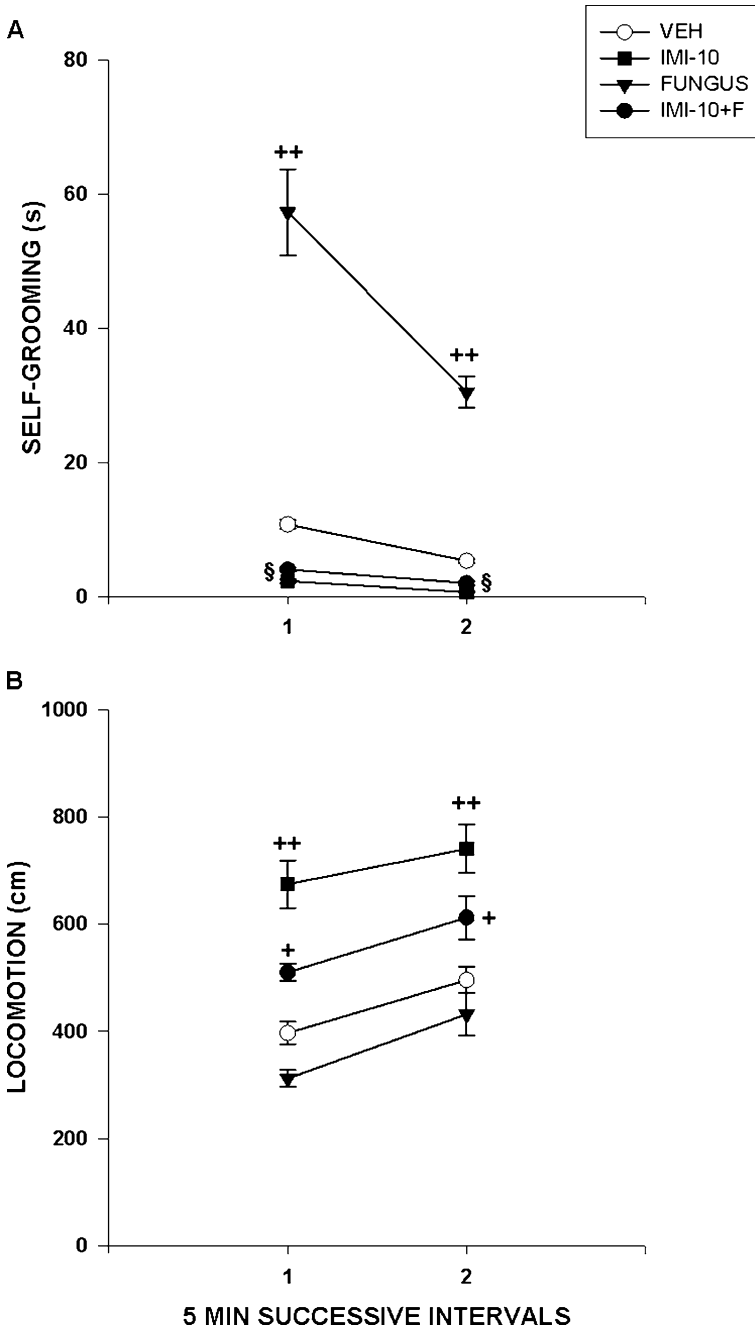
Fig. 4 Means and S. E. M. of effects of administration of IMI-10, exposure to fungal conidia and IMI-10+ F treatments on self-grooming **A** and locomotion **B**. ++ denotes significantly higher behavioral activity than the other groups. + denotes higher behavioral activity than the VEH and fungal conidia groups. § denotes lower behavioral activity than the other groups ($p<0.05$; ANOVA followed by Duncan's multiple range test). $N=24$ ants for each group

as a pro-active measure, even when not contaminated with parasites (Morelos-Juárez et al. 2010). As was also seen here for *A. subterraneus subterraneus*, exposure of *A. echinatior* workers to potential pathogens resulted in increased rates of self-grooming. However, when considering the possible protection afforded by allo-grooming, Reber et al. (2011) found no difference in the survival of *Formica selysi* exposed to *Metarhizium anisopliae* which had been maintained with or without nest-mates, assuming that the contaminated ants has been groomed in the presence of nest-mates.

In the present study a low concentration of the insecticide IMI (10 ng/insect) reliably produced both a decrease in self-grooming and an increase of locomotion behaviors of *A. subterraneus subterraneus* workers. When ants were exposed to conidial suspensions of the entomopathogenic fungus *Beauveria bassiana*, an increase in self-grooming behavior and a slight decrease in locomotion was observed. Subsequently, when the ants were exposed to IMI 10 ng/insect and fungal conidia, the decrease in self-grooming was maintained when compared to Vehicle treatments and a slight increase in locomotion was also observed. These results suggesting that the use of low concentrations of IMI and in combination with fungal conidia could be promising as a strategy for controlling leaf-cutting ants as IMI interfered with self-grooming behavior, which would be predicted to result in an increased success of the fungus in causing an infection.

In a previous study by our group (Santos et al. 2007), worker leaf-cutting ants of the species *Atta sexdens*, when exposed to low concentrations of IMI (10 ppm) together with conidial of the fungus *B. bassiana*, demonstrated a significant increase in mortality when compared to ants solely exposed to fungi. It was suggested that IMI increased the susceptibility of the ants to fungal infection by inhibiting self-grooming behavior; however behavioral modifications were not quantified by Santos and co-workers. Although Santos et al. (2007) demonstrated a synergistic effect of IMI on fungal infections, similar experiments carried out with *A. subterraneus subterraneus* workers did not show any significant differences in survival curves of fungus treated and fungus +IMI treated ants (data not shown). One of the fundamental differences in the experiments carried out here to those of Santos et al. (2007) was the insecticide administration technique. Here we applied exact doses of IMI directly to the integument, whereas Santos and co-workers exposed ants to IMI impregnated filter paper.

The dose–response curve for IMI at concentrations of 10, 20 and 40 ng/insect showed that only 10 ng/insect IMI increased locomotion and reduced self-grooming behavior. The exact mechanism by which IMI produces increased locomotion remains controversial since there are contradictory results concerning the effect of cholinergic agonists, such as IMI, on locomotion. For example, consistent with our results, Buhl et al. (2008) showed that carbachol (5 mmol.l^{-1}), a cholinergic agonist, increased locomotor activity, measured using fictive flight tests in locusts. Similar results were observed by Michelsen and Braun (1987) showing that unilateral microinjections of acetylcholine (40 nl, 10^{-2} M) into central parts of the brain of the honey bee produced an increase in ipsilateral circling (index of locomotor activity). Lambin et al. (2001) showed that the administration of IMI (5, 10 and 20 ng/insect) impaired the locomotor activity of bees, whilst concentrations of 1.25 and 2.5 ng/insect increased locomotor activity. Furthermore, locomotor and self-grooming could result in so called "behavioral competition" (Bloise et al. 2007), as ants can not perform these two acts at the same moment in time.

Behavior modification by exposure to low concentrations of insecticide could render leaf-cutting ants more susceptible to infection by entomopathogenic fungi as they fail to eliminate the conidia from their integuments, which under normal circumstances are quickly removed by efficient self-grooming. We are currently investigating the use of fungi and IMI as an alternative strategy to current control methods which rely solely on the use of insecticides.

References

Bainton RJ, Tsai LT-Y, Singh CM, Moore MS, Neckameyer WS, Heberlein U (2001) Dopamine modulates acute responses to cocaine, nicotine and ethanol in drosophila. Curr Biol 10:187–194

Bloise E, Carey RJ, Carrera MP (2007) Behavioral sensitization produced by a single administration of Apomorphine: implications for the role of Pavlovian conditioning in the mediation of context-specific sensitization. Pharmacol Biochem Behav 86:449–457

Bot ANM, Ortius-Lechner D, Finster K, Maile R, Boomsma JJ (2002) Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. Insect Soc 49:363–370

Boucias DG, Stokes C, Storey G, Pendland JC (1996) The effects of imidacloprid on the termite *Reticulitermes flavipes* and its interaction with the mycopathogen *Beauveria bassiana*. Pflanzenschutz-Nachrichten Bayer 49:103–144

Buhl E, Schildberger K, Stevenson PA (2008) A muscarinic cholinergic mechanism underlies activation of the central pattern generator for locust flight. J Exp Biol 211:2346–2357

Currie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens. Proc Natl Acad Sci USA 96:7998–8002

Currie CR, Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants. Proc R Soc Lond B 268:1033–1039

Della Lucia TMC (1993) As formigas cortadeiras. Ed. Folha de Viçosa, Brasil

Della Lucia TMC (2011) Formigas cortadeiras: da bioecologia ao manejo. Ed. UFV, Viçosa, Brasil

Della Lucia TMC, Vilela EF (1993) Métodos atuais de controle e perspectivas, p. 163–190. In Della Lucia TMC (ed.), As formigas cortadeiras. Viçosa, Folha de Viçosa. 262p

Diehl-Fleig E, Silva ME, Specht A, Valim-Labres ME (1993) Efficiency of *Beauveria bassiana* for *Acromyrmex* spp. control (Hymenoptera: Formicidae). Anais Soc Entomol Brasil 22:281–285

Elbert A, Becker B, Hartwig J, Erdelen C (1991) Imidacloprid - a new systemic insecticide. Pflanzenschutz-Nachrichten Bayer 44:113–136

Febvay G, Kermarrec A (1981) Morphologie et fonctionnement du filter infrabuccal chez une attine *Acromyrmex octospinosus* (Reich) (Hymenoptera: Formicidae): role de la poche infrabuccale. Int J Insect Morphol Embryol 10:441–449

Hughes DP, Evans HC, Hywel-Jones N, Boomsma JJ, Armitage SAO (2009) Novel fungal disease in complex leaf-cutting ant societies. Ecol Entomol 34:214–220

Lambin M, Armengaud C, Raymond S, Gauthier M (2001) Imidacloprid induced facilitation of the proboscis extension reflex habituation in the honeybee. Arch Insect Biochem Physiol 48:129–134

Little AEF, Murakami T, Mueller UG, Currie CR (2006) Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus garden. Biol Lett 2:12–16

Mattoso TC, Moreira DDO, Samuels RI (2011) Symbiotic bacteria on the cuticle of the leaf-cutting ant *Acromyrmex subterraneus subterraneus* protect workers from attack by entomopathogenic fungi. Biol. Lett. doi:10.1098/rsbl.2011.0963

Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB (2001) Neonicotinoids: insecticides acting on insect nicotinic Aceylcholine Receptor. Trends Pharmacol Sci 22:573–580

Michelsen DB, Braun GH (1987) Circling behavior in honey bees. Brain Res 421:14–20

Morelos-Juárez C, Walker TN, Lopes JFS, Hughes WOH (2010) Ant farmers practice proactive personal hygiene to protect their fungus crop. Curr Biol 20:R553–R554

Moreira DDO, Viana-Bailez AM, Erthal M Jr, Bailez O, Carrera MP, Samuels RI (2010) Resource allocation among worker castes of the leaf-cutting ants *Acromyrmex subterraneus subterraneus* through trophallaxis. J Insect Physiol 56:1665–1670

Oi DH, Pereira RM (1993) Ant behavior and microbial pathogens (Hymenoptera:Formicidae). Flor Entomol 76:63–74

Reber A, Purcell J, Buechel SD, Buri P, Chapuisat M (2011) The expression and impact of antifungal grooming in ants. J Evol Biol 24:954–964

Santos AV, Dillon RJ, Dillon VM, Reynolds SE, Samuels RI (2004) Ocurrence of the antibiotic producing bacterium *Burkholderia* sp. in colonies of the leaf-cutting ant *Atta sexdens rubropilosa*. FEMS Microbiol Lett 239:319–323

Santos A, Oliveira BL, Samuels RI (2007) Selection of entomopathogenic fungi for use in combination with sub-lethal doses of Imidacloprid. Mycopathol 163:233–240

Wilson EO, Regnier FE (1971) The evolution of the alarm-defense system in the Formicine Ants. American Nat 105:279–289