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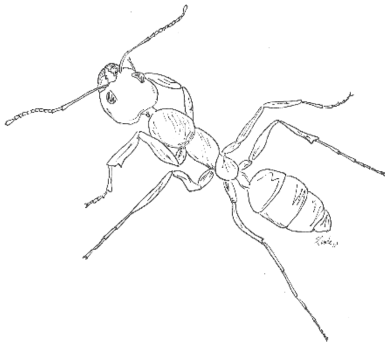

Antagonistic interactions between dominant invasive and native ant species in citrus orchards

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"We have learned from fables that ants are the model of laborious animals. In fact, much of his work is of a gravedigger. They work ceaselessly in the invisible "cemeteries" that cover the epidermis of our planet. These "gravediggers" are, at the same time, nurseries of infinite procreation, places of hidden alchemy: in them the remains of mortals become immortal, incorporated into the eternal cycles of organic matter. To ants we owe, therefore, more than the humanizing lesson of fables. This army of 100 000 000 000 000 000 individuals (one million ants for every human being) over more than 50 million years ago that converts terrestrial soil into a living and life-giving organism."

Preface by Mia Couto, *Uma Janela para a Eternidade*, by Edward O. Wilson, 2015

"People need insects to survive, but insects do not need us. If humankind were to disappear tomorrow, it is unlikely that a single insect species would go extinct, except three forms of human body and head lice…But if insects were to vanish, the terrestrial environment would soon collapse into chaos".

Edward O. Wilson (2006) *Ant Ecology*, pp. 59

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In first place I would like to thank my parents, who relentlessly provided my way here, always ready to help me under any circumstances. The ones who helped me to grow up, taught me so many things and with whom I always keep learning.

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To my grandfather that I miss so much.

To my grandmother, for her joy of living.

Abstract

The Argentine ant, *Linepithema humile* (Mayr) established in the South of Portugal (Algarve), about 120 years ago. Zina et al. (2017) compared the composition of ant communities foraging in tree canopy in citrus orchards among the three ecological subregions of Algarve (*Litoral*, *Barrocal* and *Serra*) and observed that the invasive dominant species *L. humile* was absent from *Serra*. In this work, we tested the hypothesis that dominant native ant species, such as *Tapinoma nigerrimum* (Nylander) and *Lasius grandis* Forel could prevent the Argentine ant from invading *Serra*. Laboratory experiments were carried out, using both Petri dish arenas and cages to assess the antagonistic interactions between the Argentine ant and two dominant native species, at the individual and colony level, respectively. Overall, our results support the tested hypothesis. At the individual level, both *T. nigerrimum* and *L. grandis* showed higher aggression and survival levels than Argentine ant. At the colony level, the results suggest that both the Argentine ant and *T. nigerrimum* were able to recruit a relative large number of individuals from the colony when trying to colonise a food resource defended by the competitor species. In our experimental conditions, *T. nigerrimum* showed to be more efficient than Argentine ant in this type of competition, as it was able to defend a food resource in four out of five times from the attack of the former species, as well as to dominate a resource defended by Argentine ant in four out of five times.

Key words: Competition; Invasive species; *Lasius grandis*; *Linepithema humile*; *Tapinoma nigerrimum*.

Resumo

A formiga-argentina, *Linepithema humile* (Mayr) estabeleceu-se no Algarve há cerca de 120 anos. Zina et al. (2017) compararam a composição das comunidades de formigas associadas a pomares de citrinos, nas três sub-regiões ecológicas do Algarve (Litoral, Barrocal e Serra) e verificaram que *L. humile* não estava presente na Serra. Neste trabalho, avaliou-se a hipótese de que espécies dominantes de formigas nativas, como *Tapinoma nigerrimum* (Nylander) e *Lasius grandis* Forel, poderiam impedir a formiga-argentina de invadir a sub-região Serra. Foram realizados ensaios, em placas de Petri e gaiolas para estudar as interações antagonistas entre a formiga-argentina e as duas espécies nativas dominantes, ao nível individual e da colónia, respetivamente. Em termos globais, os resultados suportam a hipótese estudada. Ao nível individual, as duas espécies de formigas nativas apresentaram maior agressividade e taxa de sobrevivência do que a formiga-argentina. Ao nível da colónia, os resultados sugerem que a formiga-argentina e *T. nigerrimum* conseguiram recrutar grande número de indivíduos, ao tentar colonizar um recurso alimentar defendido pela espécie competidora. Nas condições experimentais, *T. nigerrimum* mostrou ser mais eficiente do que a formiga-argentina neste tipo de competição, tanto na defesa de um recurso alimentar, como no domínio de um recurso defendido pela espécie competidora.

Palavras-chave: Competição; Espécies invasoras; *Lasius grandis*; *Linepithema humile*; *Tapinoma nigerrimum*.

Resumo alargado

Algumas espécies de formigas podem causar estragos em citrinos ou serem fatores de nocividade, ao favorecerem certas pragas, nomeadamente pelas relações mutualistas que estabelecem com insetos picadores-sugadores que excretam melada (Hemiptera, Sternorrhyncha), como cochonilhas, afídeos, mosquinhas brancas e psilas. Ao alimentarem-se da melada, as formigas protegem estes insetos dos potenciais predadores e parasitóides, afetando, desse modo, a sua limitação natural.

A nível mundial, existem 18 subfamílias de formigas, incluindo 335 géneros e mais de 15 300 espécies/ subespécies (Shattuck, 2017). Lach et al. (2010) sugere que a diversidade de formigas pode exceder as 25 000 espécies. Segundo Haney (1989), existem 295 espécies de formigas referenciadas em citrinos, incluindo 62 géneros e 6 subfamílias. No Algarve, Zina et al. (2017) identificaram, em citrinos, 21 espécies, incluindo 12 géneros.

Zina et al. (2017) compararam a composição das comunidades de formigas associadas a pomares de citrinos nas três sub-regiões ecológicas do Algarve (Litoral, Barrocal e Serra) e observaram que a formiga-argentina, *Linepithema humile* (Mayr), espécie invasora dominante, não estava presente na Serra, apesar ter invadido a região há cerca de 120 anos.

Neste trabalho, avaliou-se a hipótese de que espécies dominantes de formigas nativas, como *Tapinoma nigerrimum* (Nylander) e *Lasius grandis* Forel, podem impedir a formiga-argentina de invadir a sub-região Serra. Foram realizados ensaios, em placas de Petri e gaiolas para estudar as interações antagonistas entre a formiga-argentina e as duas espécies nativas dominantes, ao nível individual e da colónia, respetivamente.

Em novembro de 2016 foram recolhidos três ninhos, um de cada espécie de formiga, em pomares de citrinos previamente estudados por Zina et al. (2017) na região de Faro. Tentou-se recolher pelo menos uma rainha e o máximo de larvas e pupas, juntamente com as obreiras. Estes ninhos foram mantidos com o substrato original, em laboratório a 21 ± 1˚C, 50 – 60% r.h, em gaiolas acrílicas, previamente preparadas com Fluon™.

Os ensaios tiveram duas abordagens diferentes: Interação individual, que consistiu em colocar dois indivíduos de diferentes espécies numa caixa de Petri durante cinco minutos (combinações possíveis: *L. humile* x *T. nigerrimum*; *L. humile* x *L. grandis*; e *L. grandis* x *T. nigerrimum*) e registar o comportamento/ agressividade e atividade dos indivíduos; Interação entre colónias de *T. nigerrimum* e *L. humile* na competição por um recurso alimentar. Em cada repetição, uma das espécies foi colocada em contacto, através de um tubo transparente de 20 cm, com uma gaiola com recurso alimentar (batatas abrolhadas infestadas com cochonilha-algodão-dos-citrinos, *Planococcus citri* (Risso)). Passadas 48 h, foi introduzida a segunda espécie, ligando-a ao lado oposto da gaiola e observando se era, ou não, capaz de desalojar a espécie previamente instalada, durante um período de 1h. Foi registado o tempo de chegada ao tubo, de entrada na gaiola com recurso alimentar, da chegada efetiva ao recurso, o número de indivíduos na gaiola com recurso alimentar e o fluxo de formigas no tubo condutor.

Em termos globais, os resultados suportam a hipótese estudada. Ao nível individual, as duas espécies de formigas nativas apresentaram maior agressividade e taxa de sobrevivência do que a formiga-argentina. Ao nível da colónia, os resultados sugerem que a formiga-argentina e *T. nigerrimum* conseguiram recrutar grande número de indivíduos, ao tentar colonizar um recurso alimentar defendido pela espécie competidora. Nas condições experimentais, *T. nigerrimum* mostrou ser mais eficiente do que a formiga-argentina neste tipo de competição, tanto na defesa de um recurso alimentar, como no domínio de um recurso defendido pela espécie competidora.

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Introduction

Importance of citrus in the Mediterranean basin and Portugal

Citrus, including different plant species, such as sweet orange, mandarins, lemons, lime, and grapefruit, are among the most important fruit crops in the world, with a production of about 121 million tons per year (FAO, 2016). Native of Asia (China and India), citrus have adapted well in many areas of the world, with Mediterranean or subtropical climates, including the Mediterranean basin, North (California and Florida) and South America, Southern Africa and Australia. These fruits are present in the diet of the peoples living on the Mediterranean basin, at least since the time of the Roman Empire. In the XIX century, citrus became the main crop in various agricultural areas of the Mediterranean, playing an important role in the landscape, in the diet of the population, and as a commodity in the international trade (Duarte, 2012; Duarte et al., 2016).

According to Maria do Carmo Martins, secretary general of the National Horticultural and Operational Technology Center, COTHN (Clara, 2016), the citrus orchards occupy about 17 thousand hectares and are distributed by 24.8 thousand farms. The orange tree is the most common species, occupying 83% of the area, followed by mandarin with 12%. Algarve is the main citrus producing region (GPP, 2016), with 68% of the areas of orange trees and 80% of mandarins (Clara, 2016). In Portugal the citrus production is over 301 000 tones, with a surface over 20 000 ha (GPP, 2016; INE, 2016).

Citrus pests

Pests may damage citrus production directly, for example, by feeding on leaves, roots, or fruits, or indirectly, by serving as vectors to plant pathogens or excreting honeydew, which may soils the fruits and favour the development of sooty mold. Citrus pests include insects, mites, snails, nematodes, and some vertebrates (e.g., rodents, birds) (Berk, 2016).

Insect citrus pests are a diverse group of species dominated by sap-sucking hemipterans, such as scale insects, aphids, whiteflies and psyllids, but also including thrips, caterpillars, fruit flies, grasshoppers and ants (Berk, 2016).

Ants and sap-sucking hemipterans

Ants (Hymenoptera, Formicidae) have been studied since ancient times, and the general literature on ants is quite extensive. The largest portion of these publications deal with the physiology, behavior and/or ecology of ants in primarily non-agricultural environments, or in the laboratory. Very few, if any, species of ants are obligately associated with cultivated crops, including citrus. However, when agricultural crops displace native vegetation, a complex of otherwise unrecognized or innocuous ant species may become highly successful in monoculture cropping systems that often support a complex of pest species, usually sap-sucking hemipterans (Haney, 1989).

Sap-sucking hemipterans represent more than 50% of citrus insect pests in Portugal. As phloem feeders, they excrete honeydew, a sugar-rich food source for many insect species, including ants (Franco et al., 2000). The mutualistic interactions established between honeydew-producing insects and ants, in which ants often defend their food source against natural enemies, may favour outbreaks of honeydew-producing pests. In this interaction, ants may alter the abundance, diversity and community structure of predators and parasitoids (Calabuig et al., 2015). The relationships between ants and honeydew-producing insects have been shown to be mutually beneficial. Frequently, ants provide protection against natural enemies, unfavourable environmental conditions and contamination by the honeydew. Sapsuckers tended by ants can ingest higher quantities of phloem sap (Franco et al., 2000).

More than 60 mealybug species (Pseudococcidae), distributed among 19 genera, have been referenced in citrus at a worldwide scale. However, only a few are considered of economic importance in citrus agro-ecosystems. The citrus mealybug, *Planococcus citri* (Risso) is one of the species that most frequently assumes pest status in citrus crops, including Portugal (Franco et al., 2000, 2004).

Argentine ant

The Argentine ant, *Linepithema humile* (Mayr) is a dominant invasive species that disrupts the balance of natural ecosystems by displacing indigenous ant species throughout its introduced range (Buczkowski & Bennett, 2007). This species has spread to every single continent in the world except Antarctica (Davies, 2014), as a result of human commercial activities in habitats usually associated with human modification (Abril & Gómez, 2011). Agressive behaviour of the two European supercolonies of the Argentine ant and the intensification of human activities over the last century towards displaced native ant species of the northeastern Iberian Peninsula (Blight et al., 2017).

The Argentine ant is one of the most devastating invaders in the world, in pairs with the big-headed ant, *Pheidole megacephala* (Fabricius), the red imported fire ant, *Solenopsis invicta* Buren, the little fire ant, *Wasmannia auropunctata* (Roger) and the yellow crazy ant, *Anoplolepis gracilipes* (Smith) (Davies, 2014; Blight et al., 2016).

Argentina ant's distribution range includes areas with Mediterranean-type climates throughout the world. In the specific case of the Iberian Peninsula, it is found over the entire coastal band (Abril & Gómez, 2011) and the earliest records in Europe are from Portugal in the end of XIX century (Wetterer et al., 2009; Queiroz & Alves, 2016).

In its introduced range, the Argentina ant has impacted native faunas leading to changes in arthropod communities (Abril & Gómez, 2011), vertebrate communities and plant communities (California Academy of Science, 2017). Its presence has also negative effects on crops and plantations due to its mutualistic interactions with hemipterans, which reduce plant grow and production (Abril & Gómez, 2011).

As most invasive species, there are several characteristics that contribute to its distribution success. One is unicoloniality, an extreme case of polydomy and polygyny in ants. Unicolonial ants have huge colonies formed by a complex network of hundreds or thousands of nests, each with multiple queens. This allows the Argentine ant creating enormous aggregations of nests that are non-aggressive among each other (supercolonies) and can range over thousands of kilometres (Abril & Gómez, 2011; Blight et. al, 2017). This kind of formation may result from mixing of genetically homogenous and nonaggressive colonies, or initially aggressive colonies harbouring the most common recognition alleles (Vásquez & Silverman, 2008).

According to Abril & Gómez (2011), it is widely recognised that the European population of the Argentine ant consists of two supercolonies: the main supercolony, which follows the entire coastal band from Italy to the Iberian Peninsula; and the Catalan supercolony, apparently restricted to the eastern part of the Iberian Peninsula, but mainly present in Catalonia. Despite the long distance that can separate nests (as many as 6 000 km in the main supercolony), there is a total absence of intraspecific aggression between workers from the same supercolony. However, there is high aggression between the two supercolonies.

Other important characteristics of dispersion of this species is the fact that, being polygynous and polydomous, and many of the nests having numerous wingless queens, the queens generally don't disperse in the winged form and colonies reproduce by budding off into new units, established by as few as ten workers and a single queen (Lentini & Verdinelli, 2012).

Competition

Competition among individuals of the same species is referred to as intraspecific competition, while competition between individuals of different species is interspecific competition. For ants, both an individual ant worker or reproductive, and an ant colony, can be regarded as 'the individual' when considering competition because ant colonies are considered superorganisms and the reproductive success of the colony, which is a function of the outcomes for individual workers and reproductives, determines the evolutionary outcome for the species (Parr & Gibb, 2010).

There are three main mechanisms through which competition may occur, acting either separately or in conjunction: interference, exploitation, and apparent competition. These mechanisms operate either directly (in the case of interference competition) or indirectly (as with exploitation and apparent competition), and apply equally to intraspecific and interspecific competition (Parr & Gibb, 2010).

According to Blight et al. (2014), in ant communities we may observe interference competition and exploitative competition. Exploitative competition involves the ability of an ant species to locate a resource quickly and recruit large numbers of workers to the resource before other ant species arrive. Interference competition involves the ability of an ant species to defend a resource from another one or dominate a resource by aggressively displacing the ants already at the resource.

Dominance

According to Blight et al. (2014), dominant species may affect ant assemblages, but the nature and the intensity of such effects are species and scale dependent. For example, dominant ants regulate small-scale diversity by competition but *T. nigerrimum* may only affect species distribution, having no apparent effect on community composition.

According to Parr & Gibb (2010), in ant ecology and in the context of competition, dominance can be defined variously as behavioural, numerical, or ecological. Behavioural dominance is commonly determined using observations of interspecific interactions at food baits. Species that exhibit aggressive behaviour that causes other species to retreat or avoid them are considered behavioural. Aggressive behaviours of ants include charging, biting (most often legs or antennae), and spraying noxious chemical compounds on a competitor. The relative behavioural dominance of different species can be compared by calculating an overall dominance score or index. Territoriality in ants is also associated with behavioural dominance because territorial ants aggressively defend not only food resources and nests, but also mutually exclusive territories. Well known examples of such species include the epigaiec wood ants of the boreal region (*Formica rufa* group), meat ants in Australia (*Iridomyrmex purpureus* group), and arboreal territorial dominant species including weaver ants (*Oecophylla* spp.) in tropical forests.

Numerical dominance refers broadly to dominance due to greater numbers, biomass, and/or frequency of occurrence, and is often, but not exclusively, used with reference to baits. Although when used broadly in ecology it refers to abundance, in the context of competition it has a wide range of definitions. Usually, several measures of numerical dominance are considered simultaneously in order to provide an overall indication of dominance. Ecological dominance refers to those that have a higher abundance at baits, relative to that in pitfall traps, is thus a ratio of foraging success to general abundance in the environment (Parr & Gibb, 2010).

Ant communities associated with citrus orchards

Worldwide there are 18 subfamilies with 335 genera and over 15 300 species/ subspecies (Shattuck, 2017). Lach et al. (2010) suggests that the total diversity of ants could well exceed 25 000 species.

About 408 ant species are known from the Iberian Peninsula, distributed among 78 genera and 13 subfamilies (Sarnat et al., 2013). According to Janicki et al. (2016), there are 143 native ant species in Portugal mainland.

In his review, Haney (1989) listed 295 ant species referenced in citrus worldwide, including 62 genera and 6 subfamilies. In Portugal, Zina et al. (2017) compared the composition of ant communities foraging in tree canopy in citrus orchards among the three ecological subregions of Algarve (*Litoral*, *Barrocal*, and *Serra*). A total of 21 ant species were identified, including 12 different genera: three species from the subfamily Dolichoderinae (*L. humile*, *T. nigerrimum*, *Tapinoma simrothi* Krausse), 10 from Formicinae (*Camponotus gestroi* Emery, *Camponotus foreli* Emery, *Camponotus lateralis* Olivier, *Camponotus micans* Nylander, *Camponotus sylvaticus* Olivier, *Formica cunicularia* Latreille, *Lasius brunneus* Latreille, *L. grandis*, *Plagiolepis pygmaea* Latreille, *Plagiolepis schmitzii* Forel), seven from Myrmicinae (*Aphaenogaster senilis* Mayr, *Crematogaster auberti* Emery, *Crematogaster scutellaris* (Olivier), *Crematogaster sordidula* Nylander, *Messor structor* Latreille, *Pheidole pallidula* Nylander, *Tetramorium semilaeve* André) and one species from Ponerinae (*Hypoponera eduardi* Forel). Zina et al. (2017) observed that the invasive dominant species *L. humile* was absent from *Serra*. Considering that Algarve was invaded by *L. humile* over a century ago, it is reasonable to believe that the absence of this invasive ant species in *Serra* is not due to lack of time for its establishment. Zina et al. (2017) suggested that the higher ant richness observed in *Serra* and the absence of Argentine ant from this subregion are probably linked. They proposed three not mutually exclusive hypothesis that may explain this result: a) *Serra* subregion may be outside the ecological niche of *L. humile*; b) Invasibility of *Litoral* and *Barrocal* is facilitated by anthropogenic factors; c) The composition of ant community in *Serra* may prevent the colonization by *L. humile*.

Pest status of ants in citrus

Some of the described ants in citrus habitats are of economic importance, either by causing direct damage on citrus or inducing pest outbreaks (due to mutualistic interactions with honeydew producing insects), or also by predating citrus pests (Zina et al., 2017). Eight species of ants are considered occasional citrus pests in the Mediterranean: *Camponotus nylanderi* Emery, *C. scutellaris*, *Lasius niger* (Linnaeus), *L. grandis*, *L. humile*, *P. pallidula*, *T. nigerrimum* and *T. simrothi* (Franco et al., 2006; Pekas et al., 2011; Calabuig et al., 2015; Martínez-Ferrer & Campos-Rivela, 2017).

Objectives

The present study was aimed at testing the third hypothesis proposed by Zina et al. (2017), i.e., the composition of ant community in *Serra* may prevent the colonization by *L. humile*, in particular the interaction with dominant native ant species, such as *T. nigerrimum* and *L. grandis* (Tab. 1). The hypothesis was tested based on laboratory experiments, using both Petri dish arenas and cages to assess the antagonistic interactions between the alien Argentine ant and two dominant native species, at the individual and colony level.

Table 1 | Principal characteristics of the three studied species.

CHARACTERISTIC	ANT SPECIES		
	Linepithema humile	*Tapinoma nigerrimum*	*Lasius grandis*
SUBFAMILY	Dolichoderinae	Dolichoderinae	Formicinae
POLYMORPHISM	Monomorphic	Polymorphic	Monomorphic
FUNCTIONAL GROUP	Invasive or Exotic (IE) (Roig & Espadaler, 2010)	Generalists or Opportunists (GO) (Roig & Espadaler, 2010)	Cold climate specialists/ Shade habitats (CCS/SH) (Roig & Espadaler, 2010)
SIZE	Queen: 4 - 6 mm; Workers: 1,7 - 2,5 mm (Guerrero et al., 2012)	Queen: 5 - 6 mm; Workers: 2,4 - 3,25 mm (Sebesta, 2017; Colingwood & Prince, 1998)	Queen: 9 mm; Workers: 3 - 9 mm (Sebesta, 2017)
COLONY SHAPE	Polygynous (Collingwood & Prince, 1998)	Monogynous/ Polygnous (Sebesta, 2017)	Monogynous (Sebesta, 2017)
NEST STRATEGY	Polydomy (Lannan, 2014)	Polydomy (Lannan, 2014)	Lack of reports in literature (Lannan, 2014)
NEST SIZE	Complex network of hundreds of thousands of nests (Abril & Gómez, 2011)	A few 10 000 individuals (Sebesta, 2017)	Up to 40 000 individuals (Miner, 2014)
OVIPOSITION	28°C (Abril et al., 2008)	Nest: 21 - 24°C (Sebesta, 2017)	Nest: 21 - 24°C (Sebesta, 2017)
FORAGING	≈34°C; 15° - 30°C (Holway et al., 2002; Markin, 1970)	Arena: 18 - 28°C (Sebesta, 2017)	Arena: 18 - 28°C (Sebesta, 2017)
RESOURCE COLLECTED	Small prey, dead insects, honeydew, extrafloral nectar (Lannan, 2014)	Small and large preys, dead insects, eliasomes, honeydew (Lannan, 2014)	Small prey, honeydew (Lannan, 2014)
COLOUR	Light to dark brown (AntGuide, 2017)	Brownish black or black (AntGuide, 2017)	Yellow to dark brown (AntGuide, 2017)
NATIVE	Subtropical South America (Wetterer, 2009)	Ibero-Mauritanian (Berville et al., 2013)	Portugal, Spain, France, Morocco, Algeria, Balearic Islands (Janicki, 2016)
DISTRIBUTION	Afrotropical Region, Australasia Region, Indomalaya Region, Nearctic Region, Neotropical Region, Oceania Region, Palearctic Region (California Academy of Science, 2017)	Afrotropical Region, Indomalaya Region, Palearctic Region (California Academy of Science, 2017)	Afrotropical and Palearctic Region (California Academy of Science, 2017)

Material and Methods

Sampling and maintenance of laboratory colonies

Nests of *L. humile*, *L. grandis* and *T. nigerrimum* were sampled in 3 November 2016, from citrus orchards in the South of Portugal, Algarve (Tab. 2, Fig. 1).

Figure 1 I Collecting of the ant colonies in Algarve (SConde).

The surveyed orchards were selected based on previous knowledge on the presence of the three ant species in citrus orchards of the region (Zina et al., 2017; Vera Zina, unpublished data). In each nest, we tried to collect at least one queen and the maximal number or larvae and pupae. The collect specimens and nest soil were transported to the laboratory within containers previously coated with Fluon™.

Table 2 I Location and characteristics of the sites where ant colonies were collected.

Collected Species		*Linepithema humile*	*Tapinoma nigerrimum*	*Lasius grandis*
Region		Faro	Faro	Faro
Local		Estói	São Pedro	São Pedro
Plot		Vale Mouro	Patacão	Braciais
Coordinates	Latitude	37.087453°N	37.048758°N	37.055472°N
	Longitude	-7.887039°W	-7.948075°W	-7.950708°W
Orchard characteristics	Tree species	*Citrus sinensis*	*Citrus sinensis*	*Citrus sinensis*
	Variety	--	Newhall	Valencia Late
	Area (m²)	--	3.000	22.000

The obtained ant colonies were maintained in the laboratory at 21 ± 1˚C, 50 – 60% r.h. in translucid acrylic cages (30 - 50 cm x 30 cm x 30 cm), coated with Fluon™, within the original substrate and provided with water, sugary solution (water and sugar), and protein diet, including pollen, larvae of *Tenebrio molitor*, adults of *Drosophila* spp., boiled eggs and tuna (Fig. 2, 3).

Figure 2 I *Tapinoma nigerrimum* (Nylander) feeding on a drop of water (left) and tuna (right) (SConde).

Figure 3 | Cages in which the collected ant colonies were maintained in the laboratory (SConde).

Mealybug rearing

The citrus mealybug used in the bioassays were obtained from a population collected in citrus orchards in Silves, Portugal in sweet orange host plant and maintained in the laboratory since 2004 with regular introduction until august 2012. The insects were reared in climatic chambers (24 ± 1.0°C, 60 ± 5% r.h., in total darkness) in plastic containers with potato sprouts (*Solanum tuberosum* L.).

Experiments

Ant interactions were studied following two different approaches. In the first approach, we intended to study individual interactions by exposing one individual from each ant species, according to the three possible combinations: 1) *L. humile* x *T. nigerrimum*; 2) *L. humile* x *L. grandis*; and 3) *L. grandis* x *T. nigerrimum*. In the second approach, we studied the competition between the native *T. nigerrimum* and the alien *L. humile,* at the colony level, by investigating the ability of *T. nigerrimum* in defending or invading a food resource. All experiments were carried in laboratory conditions (19 - 25°C, 44 - 67% r.h., natural light), between November 2016 and June 2017.

a) <u>Individual interactions</u>

The experiment was carried out in glass Petri dish arenas (9 cm of diameter), coated with Fluon™, aiming to investigate the outcome of the competition, at the individual level, between Argentine ant and the native dominant species *T. nigerrimum* and *L. grandis*. In each trial, the behaviour of the two individual ant workers was monitored by both direct observation and videotape, during five minutes (Fig. 4). The type of behavioural interactions between workers was registered using the following notation adapted from Suarez et al. (1999) and Blight et al. (2010): 1 = avoid, contacts that resulted in one or both ants retreating in opposite directions; 2 = contact: antennation; 3 = aggression (biting, chemical defence, during less than two seconds); 4 = prolonged aggression between individuals, including death. An aggression score using the maximum aggression level of each species per replicate was used to compare differences in species aggressiveness and it was calculated for each ant species and combination based on the following formula: $I = (\text{-}1 \times f_1) + (1 \times f_2) + (2 \times f_3) + (3 \times f_4)$, in which f_i corresponds to the frequency of a particular type of behavioural interaction in each bioassay (adapted from Finlayson et al., 2009).

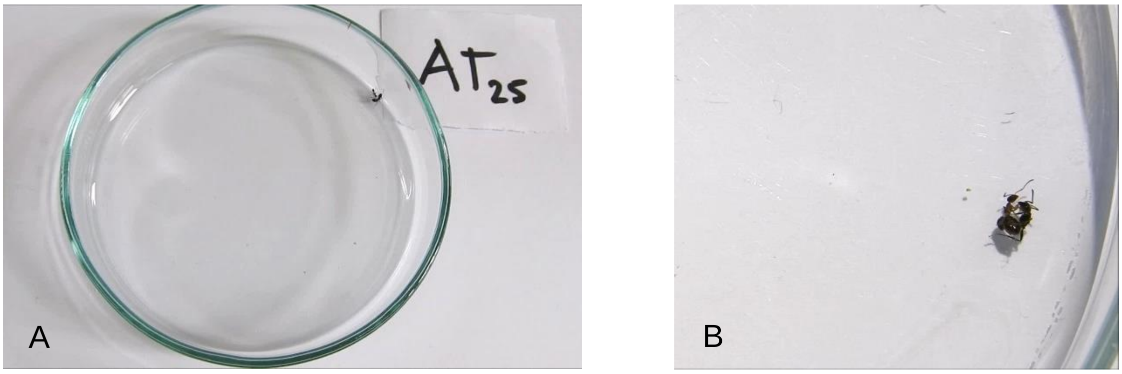

Figure 4 I Petri dish trials. A – Interaction between *Linepithema humile* (Mayr) and *Tapinoma nigerrimum* (Nylander); B – Detail of an interaction between *L. humile* and *Lasius grandis* Forel (SConde).

The activity along the observation period was also registered: very active (++); active (+); no activity (-). After each assay, the ants were individually transferred into new Petri dishes (6 cm diameter), the respective covers were sealed with parafilm or adhesive tape, and recorded the outcome (alive, injured, moribund or dead) by the end of the 5 min trial, 1 h and 24 h after the end of the trial. Subsequently, each specimen was preserved in ethanol 96% within *Eppendorf* tubes for posterior measurements. The trials for each combination of ant species were replicated 50 times, with different individuals and new Petri dishes. Due to handling errors such as escape of individuals, 40 replicates were used for *L. grandis* - *T. nigerrimum* interactions (LT) and 38 for both *L. humile* - *L. grandis* interactions (AL) and *L. humile* - *T. nigerrimum* interactions (AT). To determine the outcome of the interaction, i.e., the species performance in each replicate, a score was calculated for each species, according to the following equation:

$$\sum (a + b + c)$$

with

a = maximum level of aggression

b = mean activity

c = survival index at 5 min, 1 h and 24 h (alive, injured, moribund and dead individuals were classified as 3, 2, 1 and 0, respectively).

The species with the higher score was classified as the "winner", i.e. the species showing the best performance in each replicate.

b) <u>Interaction between colonies</u>

In this experiment, the competition between *T. nigerrimum* and the alien *L. humile* was studied using the following design: one of the two species was allowed to colonize a food resource, CA (Control trial of *L. humile*) and CT (Control trial of *T. nigerrimum*), during a 24 h period, by connecting through a 20 cm clear flexible tube (with 20 mm diameter) the ant colony cage (50 cm x 30 cm x 30 cm) to another cage (30 cm x 30 cm x 30 cm), in which the food resource was previously placed (Fig. 5). The food resource consisted of four sprouted potatoes infested with the citrus mealybug, *P. citri* (627.9 ± 87.5 individuals). The time needed for the ants to find the exit tubing, to enter the food resource cage and to colonize the infested sprouted potatoes was registered. The number of ants present in the food resource was estimated 30 min, 1 h, 1h 30, 2 h and 24 h after the start of each trial. The flow of ants within the tube was also estimated by counting for 1 minute the number of ants passing a virtual line in the middle of the tube.

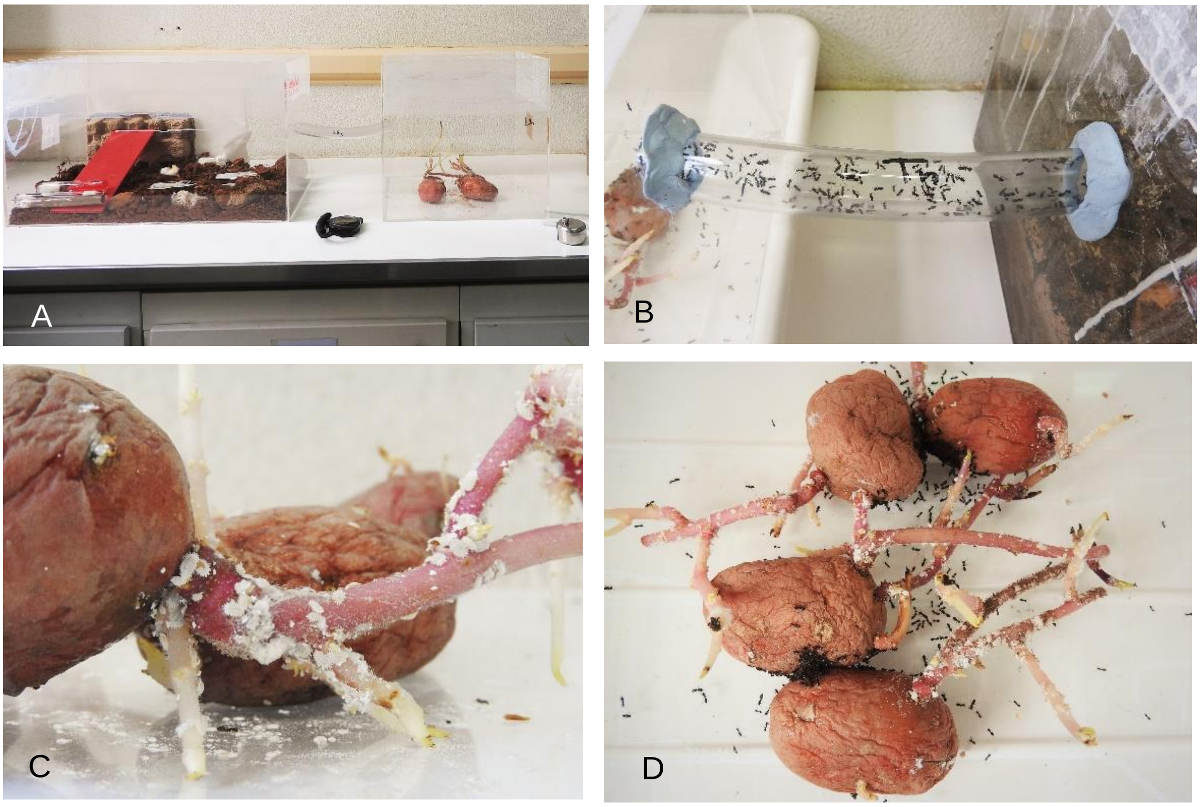

Figure 5 I Aspects of the experiments at the colony level. A – Connection between *Linepithema humile* (Mayr) colony and the food resource cage; B – Clear flexible tubing connecting the food resource cage (on the left) with *Tapinoma nigerrimum* (Nylander) colony (on the right); C – Detail of sprouted potatoes infested with citrus mealybug, *Planococcus citri* (Risso); D – *T. nigerrimum* feeding on honeydew excreted by the citrus mealybug within the food resource cage (SConde).

After the colonization period, the food resource cage was connected to the cage with the second ant species colony. Estimates of the same parameters referred before for the first ant species were also obtained for the second species. After 1 h period of exposure the result of the interaction between the two ant species was assessed by counting the number of dead, moribund, injured and alive individuals of each ant species inside the food resource cage. Two types of trials were carried out, corresponding to the two possible arrangements of the two studied ant species: 1) *T. nigerrimum* (1st species) x *L. humile* (2nd species); 2) *L. humile* (1st species) x *T. nigerrimum* (2nd species). Each trial was replicated at least five times. Due to handling errors, of all the seven replicates of BTA (Behavioural interaction between *T. nigerrimum* and *L. humile*) and six of BAT done, only five of each were used for analysis.

As we used the same colonies for all the experiments, a recovering period of at least 48 h was respected between trials to minimize possible impacts of the previous trials (Fig. 6).

Figure 6 | Design of the experiments on the interaction between ant colonies. A – *Linepithema humile* (Mayr) colony on the left, food resource cage on the centre and *Tapinoma nigerrimum* (Nylander) colony on the right; B – Detail of *T. nigerrimum* individuals' aggregation on a potato from the food resource cage; C – Detail of a track made by *L. humile* individuals within the tubing; D, E – Detail of *T. nigerrimum* foraging honeydew from the citrus mealybug, *Planococcus citri* (Risso) (SConde).

Statistical analysis

The collected data were inserted in Excel™ files and statistical analyses were performed using SPSS for Windows (IBM Corp, 2013). Differences among species in the average of maximum levels of aggression, for Petri dish trials were analysed using a t-test. A χ^2 test was used to analyse the outcome of the interactions between pairs of ant species in Petri dish trials. The result of the interaction for each replicate was based on the aggression score described before, i.e., the species with the best performance. A test was carried out for each observation period: 5 min, 1 h and 24 h.

A t-test was applied to access possible differences between the two polymorphic classes of *T. nigerrimum* workers (minor workers with body length < 3.5 mm and major workers with body length > 3.5 mm) in relation to the studied parameters.

Results

Individual interactions

a) *Behavioral interactions*

The frequency of each behavioural interaction varied among species and on the type of interaction. The most frequent behaviour was avoiding, ranging between 23.7% and 50%, except for *T. nigerrimum* in the interaction with *L. humile*, in which aggression and prolonged fight or death were more frequent. The mean of the maximal value was 3.07 for the interaction *L. humile* versus *T. nigerrimum*, 3.00 for *L. humile* versus *L. grandis*, and 2.98 for *L. grandis* versus *T. nigerrimum*. *Tapinoma nigerrimum* showed the highest mean aggression levels and aggression scores, and *L. humile* the lowest ones (**Tab. 3**). No significant differences were found among the three ants species in the maximal aggression level (**Tab. 4**).

Table 3 I Mean aggression level, aggression score and frequency of each type of behavioural interaction for each ant species and bioassay combination.

Bioassay combination	Ant species	Frequency of each type of behavioral interaction (%)				Mean aggression level ± SE	Aggression Score
		1 = avoid	2 = antennation	3 = aggression	4 = prolonged fight or death		
LT (N=40)	*T. nigerrimum*	40.0	2.5	25.0	32.5	2.88 ± 0.14	110
	L. grandis	45.0	2.5	15.0	32.5	2.68 ± 0.18	85
AT (N=38)	*T. nigerrimum*	23.7	2.6	39.5	26.3	2.74 ± 0.18	137
	L. humile	31.6	0.0	26.3	26.3	2.47 ± 0.22	100
AL (N=38)	*L. grandis*	34.2	0.0	29.0	26.3	2.61 ± 0.19	103
	L. humile	50.0	2.6	15.8	26.3	2.55 ± 0.18	63

b) *Activity*

The mean activity of ant species decreased along the 5 min trials in all treatments (**Fig. 7**). The higher activity level was registered in *L. grandis*, followed by *T. nigerrimum* and *L. humile*. The reduction on the activity seems to be dependent on the species and treatment. For example, the activity reduction showed by *T. nigerrimum* was higher when exposed to *L. grandis,* compared to that observed in the interaction with *L. humile*. No significant differences were found among ant species in mean activity (Tab. 4).

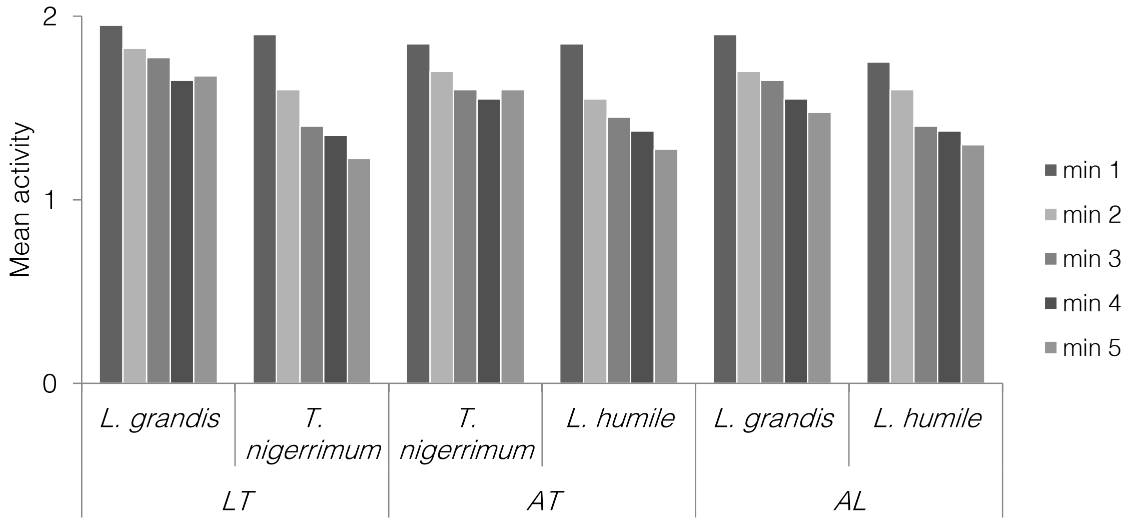

Figure 7 I Mean activity of species per minute during each trial, LT (*Lasius grandis* Forel vs. *Tapinoma nigerrimum* (Nylander)), AT (*Linepithema humile* (Mayr) vs. *T. nigerrimum*) and AL (*L. humile* vs. *L. grandis*).

c) <u>*Survival*</u>

The survival in the end of the 5 min bioassays was higher in *L. grandis* compared with *T. nigerrimum* and *L. humile* (Fig. 8). The survival rate did not decrease much up to 1 h after the bioassay for the three ant species. However, this parameter registered a sharp decrease 24 h later, reaching a similar level of survival for both *L. grandis* and *T. nigerrimum.*

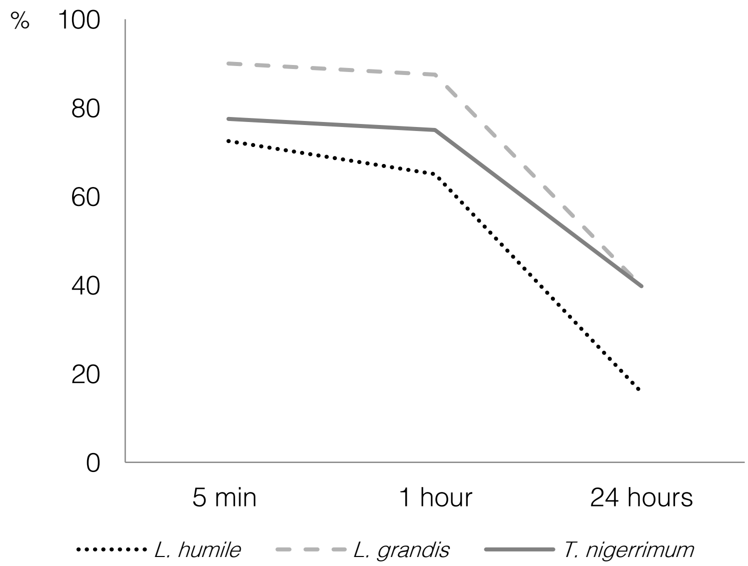

Figure 8 I Mean number of individuals (%) alive at 5 minutes, 1 hour and 24 hours after the trials.

The values of survival index at 5 min and 1h for *L. grandis* were significantly higher than those of *L. humile* (**Tab. 4**). No significant differences were observed between *L. grandis* and *T. nigerrimum* for the same parameters, as well as between the last species and *L. humile.* The survival index at 24 h for both *L. grandis* and *T. nigerrimum* was significantly higher than that of *L. humile.* No significant differences were registered between the first two ant species.

d) Outcome of the interactions

The outcome of the interactions between the three studied ant species was analysed by comparing the frequency each species won the competition with that if there was no "winners". The result was similar for both 5 min and 1 h observations (**Fig. 9**). The frequency of trials *L. grandis* won the competition with *L. humile* (χ^2=6.041, df=1, p=0.014 for both 5 min and 1 h observations), and *T. nigerrimum* (χ^2=5,263, df=1, p=0.022, for 5 min observations; χ^2=3.959, df=1, p=0.047, for 1 h observations) was significantly higher than expected. *Tapinoma nigerrimum* also won more frequently the competition with *L. humile* (χ^2=11.845, df=1, p=0.001 for 5 min observations; χ^2=23.405, df=1, p<0.001, for 1 h observations). However, for the 24 h observations, only the interaction between *L. grandis* and *L. humile* showed significant differences (χ^2=19.600, df=1, p<0.001). No significant differences were registered in the competition between *L. humile* and *T. nigerrimum* (χ^2=0.362, df=1, p=0.547), as well as between *L. grandis* and *T. nigerrimum* (χ^2=2.315, df=1, p=0.128) (**Fig. 9**).

e) Size differences among ant species

There were significant differences in the body length among ant species (F=72.738, df=2, p<0.001, **Tab. 4).** The size of *L. grandis* and *T. nigerrimum* workers were significantly higher than that of *L. humile*. No significant differences were found between *L. grandis* and *T. nigerrimum.*

Body length differed significantly between major and minor workers of *T. nigerrimum*. No significant differences were found between these two size classes of ants for mean activity, mean maximum aggression behaviour and survival index (**Tab. 5**).

Interaction between colonies

Both ant species registered a higher number of individuals when exposed to the other ant species compared to the corresponding control trials (**Fig. 10**). Comparing for each ant species the number of individuals in the control with that when trying to colonize the food resource defended by the other species, there was a 647% and 90% of increase in the case of *L. humile* and *T. nigerrimum,* respectively. The mean number of individuals in the control trials of *Tapinoma nigerrimum* was higher than that of *L. humile.*

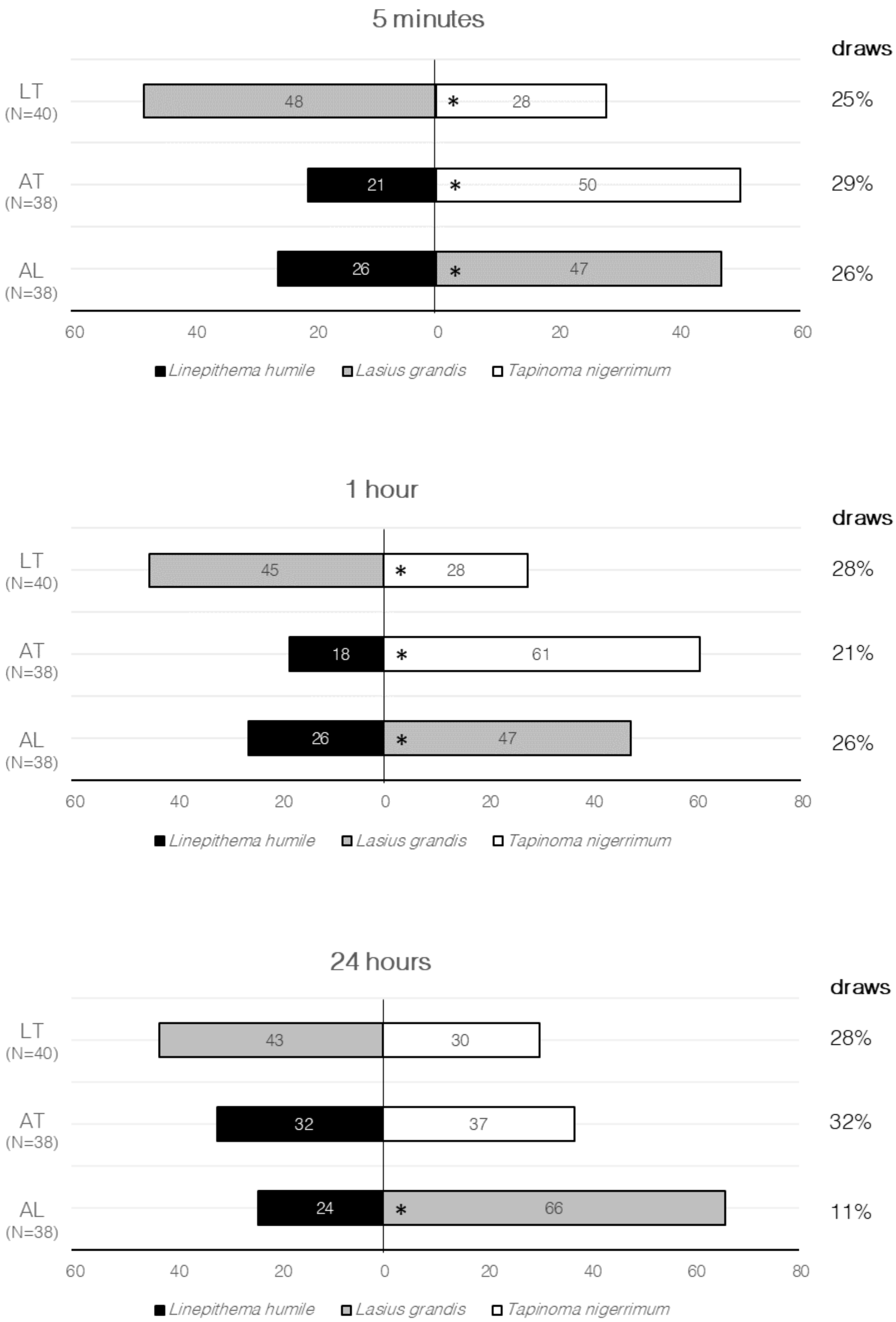

Figure 9 I Frequency (%) of trials "won" by each ant species in each modality of species interaction (combined results on behaviour, activity and survival of species at 5 minutes, 1 hour and 24 hours). AL = *Linepithema humile* (Mayr) vs. *Lasius grandis* Forel; AT = *L. humile* vs. *Tapinoma nigerrimum* (Nylander); LT = *L. grandis* vs. *T. nigerrimum*. The asterisk indicates that the observed frequency responses differed significantly (p<0.05). The number of draws are presented at the right side of the figure, for each modality.

Table 4 I Mean body length, mean activity, mean maximal aggression level and survival index of the studied ants (mean ± SE).

Parameter *	*Species*		
	Linepithema humile	*Lasius grandis*	*Tapinoma nigerrimum*
Body length	2.81 ± 0.06 [a]	3.70 ± 0.06 [b]	3.62 ± 0.06 [b]
Activity	1.54 ± 0.06 [a]	1.71 ± 0.06 [a]	1.59 ± 0.06 [a]
Maximal aggression level	2.55 ± 0.13 [a]	2.64 ± 0.13 [a]	2.81 ± 0.13 [a]
Survival index 5 min	2.44 ± 0.08 [a]	2.87 ± 0.08 [b]	2.67 ± 0.08 [ab]
Survival index 1 h	2.27 ± 0.10 [a]	2.82 ± 0.10 [b]	2.54 ± 0.10 [ab]
Survival index 24 h	0.55 ± 0.15 [a]	1.24 ± 0.15 [b]	1.31 ± 0.15 [b]

* Means followed by a different letter within a row are significantly different (p<0.05).

Table 5 I Mean body length, mean activity, mean maximal aggression level and survival index of minor and major workers of *Tapinoma nigerrimum* (Nylander).

Parameter	Size class of ant workers	N	Mean ± SE
Body length *	Minor workers	37	3.03 ± 0.05
	Major workers	41	4.15 ± 0.08
Activity	Minor workers	37	8.35 ± 1.12
	Major workers	41	7.39 ± 1.01
Maximal aggression level	Minor workers	37	2.62 ± 0.18
	Major workers	41	2.98 ± 0.15
Survival index 5 min	Minor workers	37	2.73 ± 0.09
	Major workers	41	2.61 ± 0.13
Survival index 1 h	Minor workers	37	2.59 ± 0.14
	Major workers	41	2.49 ± 0.15
Survival index 24 h	Minor workers	37	1.05 ± 0.23
	Major workers	41	1.54 ± 0.23

* Parameters marked with an asterisk are significantly different (p<0.05).

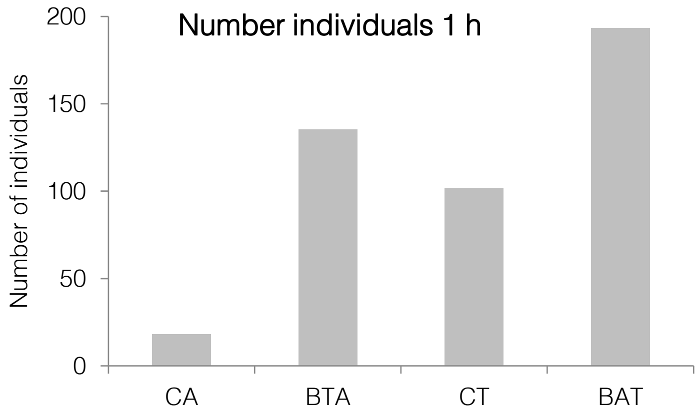

Figure 10 | Mean number of individuals inside the resource cage after 1 h trial. CA = Control trial of *Linepithema humile* (Mayr) (N=8); BTA = Behaviour trial of *Tapinoma nigerrimum* (Nylander) (1st species) vs. *L. humile* (2nd species) (N=5); CT = Control trial of *T. nigerrimum* (N=8); BAT = Behavioural trial of *L. humile* (1st species) vs. *T. nigerrimum* (2nd species) (N=5).

Linepithema humile took more time entering the tube and cage and arriving the food resource than *T. nigerrimum* (Fig. 11*Figure 11*). Both species were faster in the controls than in the presence of the other species. *Linepithema humile* did not reach the food resource in four out of five trials (and only one individual reached the resource in that trial). *Tapinoma nigerrimum* was able to colonize the resource in all five trials, except one in which the initial number of Argentine ant individuals was the highest (100 individuals, with a mean number of 24 in the other replicates). Most of the times *T. nigerrimum* was feeding actively on mealybug honeydew at the end of the trials and even 20 - 40 minutes before. The mean number of *T. nigerrimum* individuals at the beginning of trials was higher (mean number = 346, ranging from 114 to 630) than that of *L. humile* (mean number = 39.4, ranging from 22 to 100).

The relative frequency of damaged, moribund or dead individuals of *L. humile* in the end of the trials was much higher than in *T. nigerrimum* in both treatments (Fig. 12).

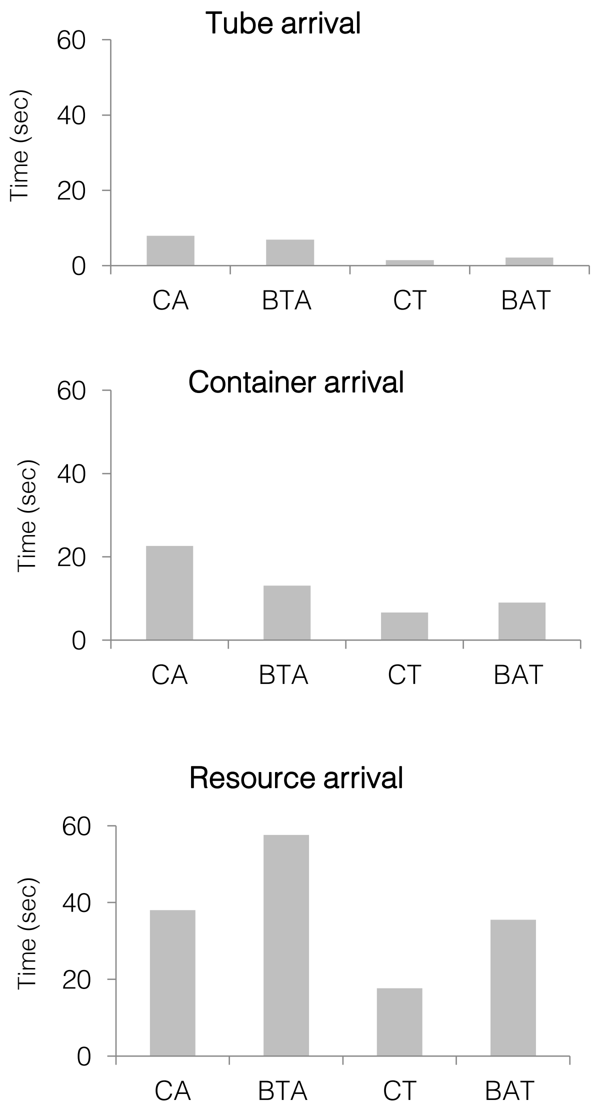

Figure 11 | Mean time (seconds) of arrival to the tube, cage and food resource for each treatment and ant species control. CA = Control trial of *Linepithema humile* (Mayr); BTA = Behaviour trial of *Tapinoma nigerrimum* (Nylander) (1st species) vs. *L. humile* (2nd species); CT = Control trial of *T. nigerrimum*; BAT = Behavioural trial of *L. humile* (1st species) vs. *T. nigerrimum* (2nd species).

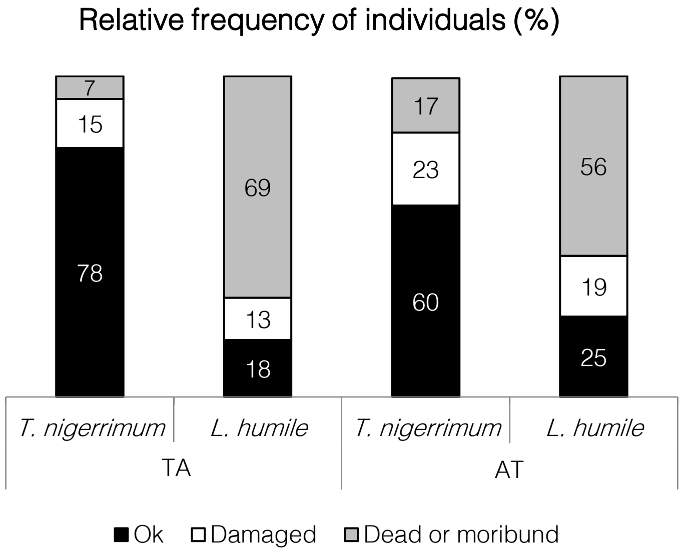

Figure 12 I Relative frequency of individuals alive, injured and dead or moribund for *Tapinoma nigerrimum* (Nylander) and *Linepithema humile* (Mayr) at the end of the trials. TA = *T. nigerrimum* vs. *L. humile*; AT = *L. humile* vs. *T. nigerrimum*.

The flow of individuals in both ant species within the tube was higher in both treatments compared to the corresponding control trials (**Fig. 13**). The flow of individuals in *T. nigerrimum* was higher than that of *L. humile* in both treatments, in particular in BTA.

The flow of individuals between the colony and the food resource increased until 2 h after the begin of the experiment, in the case of *L. humile,* and 1.5 h for *T. nigerrimum* (**Fig. 14**). This parameter maintained similar level along the rest of the 24 h period in *T. nigerrimum,* but decreased in *L. humile.* The mean values were always more than two times higher in *T. nigerrimum* than in *L. humile.*

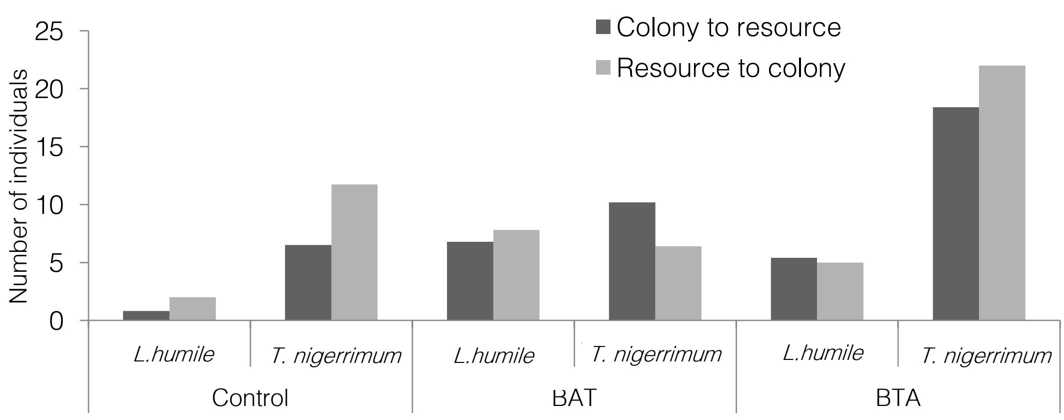

Figure 13 I Mean number of individual's flow per minute within the tube at the end (1 h) of the trials for each species and treatment. CA = Control trial of *Linepithema humile* (Mayr); BTA = Behaviour trial of *Tapinoma nigerrimum* (Nylander) (1st species) vs. *L. humile* (2nd species); CT = Control trial of *T. nigerrimum*; BAT = Behavioural trial of *L. humile* (1st species) vs. *T. nigerrimum* (2nd species).

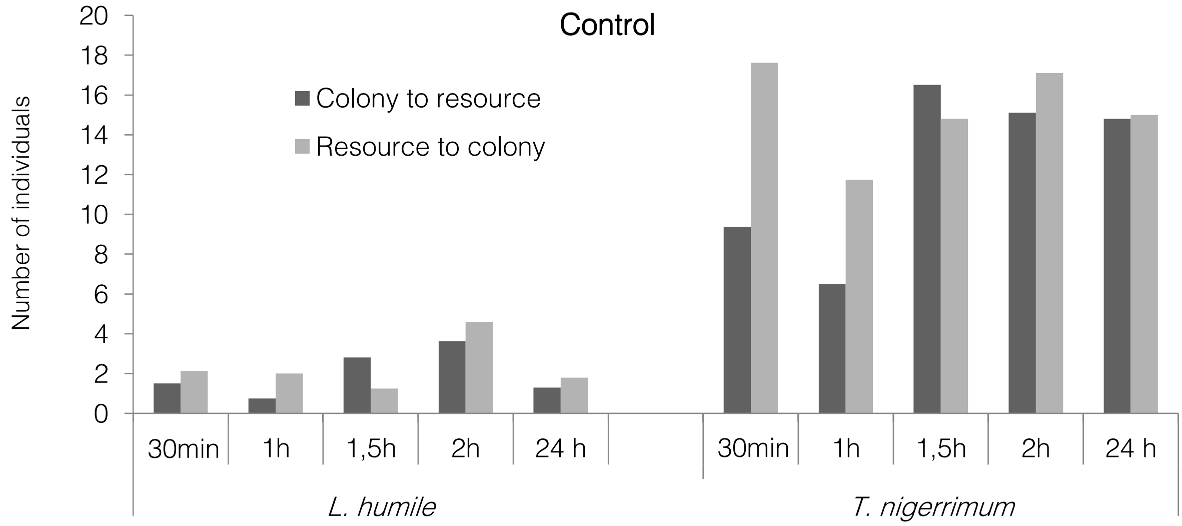

Figure 14 I Mean number of individual's flow per minute within the tube between the colony cage and food resource cage for each ant species, in the control trials, during a period of 24 h.

Discussion

Zina et al. (2017) hypothesised that dominant native ant species, such as *T. nigerrimum* and *L. grandis* could prevent the alien Argentine ant from invading the subregion *Serra* of Algarve. Here we tested this hypothesis in two types of laboratory experiments, at both individual and colony interaction levels. Overall, our results support this hypothesis. At the individual level, both native ant species showed higher aggression and survival levels than Argentine ant, in the interaction. Nevertheless, it is interesting to highlight that the frequency of avoiding behaviour was relatively high (24 - 50%), although the level was apparently dependent on the type of interaction. In general, both *T. nigerrimum* and *L. grandis* "won" the competition with *L. humile* more frequently than expected by chance. The better competitive performance showed by *L. grandis* may be at least in part related with possible differences on the impact of its chemical defences compared to those of the two Dolichoderinae species (*L. humile* and *T. nigerrimum*). In fact, Formicinae, such as *L. grandis* are known to spray their venom secreted by the venom gland, whereas Dolichoderinae spray their targets with different chemical compounds, such as ketones and iridoids secreted by pygidial glands (Touchard et al., 2016).

At the colony level, the results suggest that both the Argentine ant and *T. nigerrimum* were able to recruit a relative large number of individuals from the colony when trying to colonise a food resource defended by the competitor species. This recruitment capacity, in relative terms, seems to be much higher (about seven times higher than in *T. nigerrimum*) in the case of Argentine ant. Despite of that, the Argentine ant was not able to conquer the food resource defended by *T. nigerrimum,* whereas the opposite was true in four out of five times. However, the fact that the only occasion *T. nigerrimum* was not able to reach the food resource occupied by Argentine ant corresponded to a situation in which the Argentine ant had a higher number of individuals within the cage suggests that the outcome of the interaction between these two species may be density dependent. This hypothesis is supported by the results obtained by Holway & San Diego (2000). These authors observed that Argentine ant was able to maintain an average of 10 or more workers at baits in the presence of a competitor ant, *Forelius mccooki* (McCook), only when colonies were larger than 1 000 workers.

The design used in our experiments allowed us to investigate interference competition (the ability of an ant species to defend a resource from another one or dominate a resource by aggressively displacing the ants already at the resource (Blight et al., 2014)), between the Argentine ant and the native dominant species *T. nigerrimum.* In our experimental conditions, *T. nigerrimum* showed to be more efficient than Argentine ant in this type of competition, as it was able to defend a food resource in four out of five times from the attack of the former species, as well as to dominate a resource defended by Argentine ant in four out of five times. However, in field conditions the outcome of the interaction between competitors is also dependent on exploitative competition (the ability of an ant species to locate a resource quickly and recruit large numbers of workers to the resource before other ant species arrive (Blight et al., 2014)), which was not studied by us. Polygyny, in which Argentine ant colonies comprise up to 16.3 queens per 1 000 workers, each producing up to 60 eggs per day, and its polydomous (multiple-nest) structure, in which colony workers can move between nests, provides high worker densities to discover and defend resources (Silverman & Brightwell, 2008). Competitive performance of Argentine ant is positively associated with worker number, but the influence of nest number seems to be more complex, depending for example on the colony size (Holway & San Diego, 2000).

Possible differences among ant species in the attraction to the food resource may influence the outcome of the competitive interaction, as we only used one honeydew producing insect, *P. citri*. Future studies should consider this aspect.

The use of only one colony per species for all the trials may also influence the results, as replicates are not completely independent. Ideally, we should use a different nest per replicate. However, in practical terms this is very difficult to implement.

Conclusion

In conclusion, the results of this study support the hypothesis that dominant native ant species, such as *T. nigerrimum* and *L. grandis* may prevent the alien Argentine ant from invading the subregion *Serra*, in Algarve. At the individual level, both native ant species showed higher aggression and survival levels than Argentine ant, in the interaction. In our experimental conditions, *T. nigerrimum* showed to be more efficient than Argentine ant in the competition, as it was able to defend a food resource in four out of five times from the attack of the former species, as well as to dominate a resource defended by Argentine ant in four out of five times.

Knowledge on the factors affecting invasiveness of *L. humile* is of relevance for pest management in citrus orchards as the composition of ant communities in these agro-ecosystems may influence pest status of different insect species through their interaction with honeydew-producing hemipteran (Zina et al., 2017).

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