



Behavioral responses to numerical differences when two invasive ants meet: the case of *Lasius neglectus* and *Linepithema humile*

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Abstract Two of the world's most invasive ants, *Linepithema humile* and *Lasius neglectus*, are destined to overlap in range as they continue to spread throughout Europe. Although *L. humile* arrived first, and is therefore more numerically abundant, *L. neglectus* is the more behaviorally dominant of the two. We performed lab trials to determine whether *L. humile* could use numerical abundance to overcome the behavioral dominance of *L. neglectus* and whether the ants' behavioral patterns shifted when the species co-occurred. We found that *L. neglectus* was more aggressive when less abundant, whereas the opposite was true of *L. humile*. When *L. neglectus* was outnumbered, it employed aggressive

behaviors, such as biting or chemical attacks, more frequently than *L. humile*; it also utilized a behavioral sequence that included mandible opening and biting. Our results for these species support the hypothesis that species modulate their behavior towards competitors, which facilitates the understanding of how multiple invasive ant species can co-occur in a given area. Moreover, our study shows that the co-occurrence of invasive species could result from the use of two strategies: (1) the Bourgeois strategy, in which aggressiveness changes based on numerical dominance and (2) the dear-enemy strategy, in which aggressiveness is reduced when competitors co-occur. Since these strategies may lead to territory partitioning, we suggest that the behavioral flexibility displayed by both species when they overlap may allow local co-occurrence and increase their likelihood of co-occurrence during their range expansion in Europe, which could have a negative cumulative impact on invaded areas.

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Introduction

Invasive species are, by definition, alien species that have serious environmental, economic, and human health impacts, and they are considered to represent a global threat to biodiversity (Keller et al. 2011; Early

et al. 2016). Invasions occur in stages, which are defined by the barriers that all exotic species must surmount: first, a species must arrive in an area outside its native range; second, it must survive and establish itself in that new area; and, third, it must spread to additional areas (Blackburn et al. 2011; Leung et al. 2012). Once a non-native species has arrived in a new area, its likelihood of establishment is determined by a variety of factors, including propagule size and composition (Simberloff 2009); abiotic conditions (e.g., climatic niche matching; Abellán et al. 2017); and biotic conditions (e.g., the resistance of the native community; Jeschke 2014).

Ants are a particularly prominent group of invasive species, with five species among the selection of the 100 worst invaders (Lowe et al. 2000) and 19 recognized by the IUCN Invasive Species Specialist Group [Global Invasive Species Database (<http://www.issg.org/database>)]. Moreover, biogeographic barriers have been broken down frequently for ants, by human trade and travel (for example, more than 4 thousand border interceptions in USA and New Zealand over a period of 70 years, Bertelsmeier et al. 2018) and more than 240 alien ant species are known to have established outside their native range (Bertelsmeier et al. 2017). Because of this increased prevalence of ant invasive species (Suárez et al. 2005; Bertelsmeier et al. 2018) together with their trait similarity (e.g. small size, sociality and diversity of nesting habits, Bertelsmeier et al. 2017), many of these invasive species are coming into direct contact; this could result in their co-occurrence (e.g., Vonshak and Gordon 2015) or the displacement of one species by the other one (e.g., LeBrun et al. 2013; Wetterer 2017). Mechanisms of co-occurrence of invasive ant species are variable, including differences in microhabitat preferences (Morrison 1996; Trigos-Peral et al. 2020), behavioral differences (Morrison 1996; Holway and Suárez 1999; Axen et al. 2014; Bertelsmeier et al. 2015a) or different effectiveness in resource exploitation (Cerdá et al. 1997; Kneitel and Chase 2004; Berkley et al. 2010). Food resources can modulate the degree of aggressiveness displayed by the invasive species (Morrison 1996), which is usually mediated by a tradeoff between resource discovery and resource dominance (Zheng et al. 2008; Axen et al. 2014). Differences in aggressiveness among invasive ant species can facilitate their co-occurrence (Zheng et al. 2008; Bertelsmeier et al. 2015a, b); in

fact, since invasive species are usually characterized as highly aggressive (Bertelsmeier et al. 2015a), their behavioral plasticity (Holway and Suárez 1999) becomes crucial to their success in an habitat invaded by multiple ant species. Thus, studies focused on the behavioral mechanisms facilitating the co-occurrence of invasive ant species are fundamental to understand their potential impact on the invaded areas and predict their future distribution.

Among the invasive ant species found in Europe two are highly successful: the Argentine ant, *Linepithema humile*, and the invasive garden ant or Anatolian ant (Czechowski et al. 2012), *Lasius neglectus*. Both *L. humile* and *L. neglectus* are among the 19 species listed as highly invasive by the IUCN Invasive Species Specialist Group (Lowe et al. 2000; Global Invasive Species Database [<http://www.issg.org/database>]). Like other invasive ants, *L. humile* and *L. neglectus* display life history traits that are related to invasiveness: smaller size relative to related taxa, omnivory, mass recruitment to resources, polygyny (i.e., nests contain multiple queens), within-nest mating, budding dispersal, synanthropy (i.e., a preference for anthropogenic habitats), strong interspecific aggression, polydomy (i.e., presence of multiple interconnected nests), and unicoloniality. This latter characteristic results in low levels of intraspecific aggression, giving rise to supercolonies (Boomsma et al. 1990; Holway et al. 2002; Bertelsmeier et al. 2017). While *L. neglectus* does not seem to form distinct supercolonies in Europe (Ugelvig et al. 2008), it appears that *L. humile* has established at least three supercolonies in Europe: the Main supercolony, whose colonies are spread across the continent, as well as the Catalanian supercolony and the Corsican supercolony, whose colonies are restricted to specific locations within southwestern Europe (Giraud et al. 2002; Vogel et al. 2009; Blight et al. 2012).

These two species share ecological similarities and can therefore colonize the same types of habitats. However, they arrived at different times in Europe. *Linepithema humile* was first detected in Portugal in 1890 (Wetterer et al. 2009) and has since firmly established itself across the continent (Giraud et al. 2012). *Lasius neglectus* was first detected in Budapest in the 1970s and is continuing to spread across Europe (Espadaler et al. 2007). When the species overlap within a given area, differences in aggressiveness and/or competitive strategies could determine whether the

ants can successfully co-occur. Previous studies have found that *L. neglectus* is behaviorally more aggressive than *L. humile* (Bertelsmeier et al. 2015c), a trait that is greatly advantageous during interspecific interactions. That said, *L. humile*'s colony density and size have increased over the course of its invasion (Diaz et al. 2014). Therefore, *L. humile*'s earlier arrival in Europe means that it could be more numerically abundant and that it might already be well established when *L. neglectus* arrives at a given location, which could allow *L. humile* to exclude *L. neglectus* when the two species co-occur.

Moreover, because both species do co-occur in certain areas, we might expect changes in their competitive behavior over time. Indeed, invasive species have been observed to display behavioral shifts as a result of both genotypic and phenotypic plasticity (Axen et al. 2014; Strayer et al. 2006; Ricciardi et al. 2017). For example, *L. humile* can assess the relative abundance of competitors and respond accordingly. When competitors were present in high numbers, *L. humile* used an avoidance or escape strategy; when competitors were present in lower numbers, *L. humile* reacted more aggressively (Sagata and Lester 2009). This behavioral plasticity, in which competitor number determines a species' response, is known as the Bourgeois strategy (Maynard Smith 1982), and it could help invasive species successfully navigate the first stages of invasion, notably when they are facing numerically dominant species or other invasive species. It is therefore possible that an invasive species that has dominated a community for years can be replaced. This phenomenon, known as the "treadmill effect" (Thomas and Reid 2007), has been observed in different organisms. It appears to be most common in marine animals (Jackson 2015), but it has also been seen in some terrestrial systems. For example, in a mid-Atlantic community, mile-a-minute weed (*Persicaria perfoliata* [L.] H. Gross), an invasive Asian vine, was replaced by Japanese stiltgrass (*Microstegium vimineum* [Trin.] A. Camus), an invasive annual (Lake et al. 2011). In ants, invasive species replacements are also frequent; classic examples include the replacement of the red imported fire ant (*Solenopsis invicta*) by the imported crazy ant (*Nylanderia fulva*) in some regions of the Southeastern United States and Texas (LeBrun et al. 2013) or the replacement of the invasive *Pheidole megacephala* by *L. humile* on the islands of

Bermuda (Haskins & Haskins 1965, 1988; Lieberburg et al. 1975; Wetterer 2017).

The dear-enemy strategy is another phenomenon that helps minimize the costs associated with continuous fights between co-occurring species (Boulay et al. 2007; Dimarco et al. 2010): interspecific confrontations have been found to be less aggressive when species co-occur than when they do not. Over the long term, continual highly aggressive competition for resources is expensive (Fisher 1954), and the resulting loss of workers and energy might exceed the benefits of competitive success. Thus, the dear-enemy strategy can lead to a form of equilibrium by decreasing aggressiveness among neighboring species and thus limiting the losses sustained by interacting colonies (Christensen and Radford 2017). The adoption of this strategy by invasive species might lead to the co-occurrence of multiple invasive species in a given area, which can have different outcomes and impacts in native communities (Simberloff and Von Holle 1999; Jackson 2015).

The objective of this study was to characterize the competitive abilities of *L. humile* and *L. neglectus* during paired confrontations to better understand the mechanisms that could allow their co-occurrence, which has been observed in at least one area (X. Espadaler, personal communication). We thus wanted to discern whether their co-occurrence could be explained by the Bourgeois strategy and/or the dear-enemy strategy. First, to determine whether the Bourgeois strategy was in operation, we used lab trials in which we tested the effects of experimentally modifying species numerical dominance. If *L. humile* was employing the Bourgeois strategy, we predicted that, when numerically dominant, *L. humile* would act more aggressively toward *L. neglectus*; conversely, *L. humile* was expected to act less aggressively when numerically subordinate. Alternatively, if *L. humile* was not employing the Bourgeois strategy, we predicted that its numerical dominance would have no effect on its aggressiveness, which would allow the co-occurrence of the two species. Second, to determine whether the dear-enemy strategy was in operation, we used paired confrontations between ants from colony fragments collected in areas where the species do and do not co-occur. If the dear-enemy strategy was being employed, we predicted that the level of interspecific aggressiveness would be lower when the ants faced a familiar versus unfamiliar competitor.

The utilization of this strategy could also allow the co-occurrence of the two species.

Materials and methods

Colony collection and maintenance

In October 2016, we collected four *L. humile* colony fragments from four different locations in Spain (Doñana National Park, Seville, Girona, and Sant Cugat) and three *L. neglectus* colony fragments from two locations in Spain (Sant Cugat and Gandía) and one location in Poland (Warsaw) (Fig. 1). The *L. humile* colony fragments came from two of the species' three European supercolonies (Blight et al. 2010, 2012, 2017). More specifically, the colony fragments from Doñana, Seville, and Girona came from the Main supercolony, and the colony fragment from Sant Cugat belonged to the Catalanian supercolony.

We collected large colony fragments (hereafter, colonies) to ensure that the lab colonies would function normally, given that having insufficient numbers of workers can detrimentally affect tasks

such as foraging and territory defense (Burchill and Moreau 2016). Consequently, the lab colonies comprised 300–500 workers and at least one queen. Before the experiment began, all the colonies were maintained under the same constant conditions for 2 weeks to allow the ants time to acclimatize: we kept the room temperature at 23–25 °C, used the photoperiod associated with the month of March in Poland (sunrise about 6 a.m. and sunset about 6 p.m.), and fed the ants a standard Bhatkar-Whitcomb diet (Bhatkar and Whitcomb 1970).

Experimental design

Our experiment consisted of staging confrontations between the two ant species. The aim was to simulate situations in which one invasive species had arrived and become established before the other invasive species. To this end, in each confrontation, one ant (referred to as numerically subordinate) from one species faced five ants from the other species (referred to as numerically dominant). There were thus two types of confrontations: one in which *L. humile* was numerically dominant (HUMdom: 5 *L. humile* vs. 1 *L. neglectus*) and the other in which *L. neglectus* was

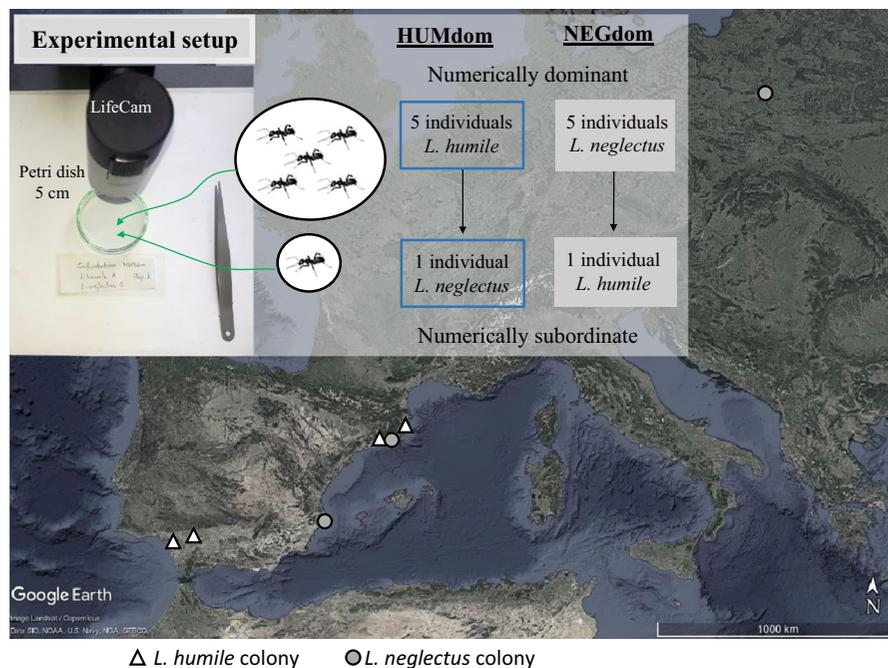


Fig. 1 Map of the locations where the colonies were collected and schematic representation of the experimental set-up. Source of map: Google Earth Pro v. 7.3.2.5776. (December 14, 2015). <http://www.earth.google.com> (image captured on January 3, 2020)

numerically dominant (NEGdom: 5 *L. neglectus* vs. 1 *L. humile*) (Fig. 1).

The confrontations took place in Petri dishes (diameter: 5 cm) whose sides had been coated with Teflon Fluon to prevent the ants from escaping. Ants were gently transported straight from their lab colonies to a Petri dish. The five numerically dominant ants were placed in the dish first and left there for 2 min to acclimatize; the numerically subordinate ant was then added, simulating the arrival of a competitor in an already colonized area. Each confrontation type (HUMdom and NEGdom) was repeated using different combinations of colonies, and each combination was repeated two times. A total of 48 confrontations were performed between the 4 colonies of *L. humile* and the 3 colonies of *L. neglectus*. There were 24 confrontations in which *L. humile* was numerically dominant (HUMdom: 12 combinations repeated twice) and 24 confrontations in which *L. neglectus* was numerically dominant (NEGdom: 12 combinations repeated twice). For each confrontation, we used new workers, which were removed from the colony afterwards. The confrontations were run for 5 min, and a video recording was made of each using a camera (Microsoft LifeCam Studio Webcam) placed 20 cm above the Petri dish (Fig. 1). We then analyzed the videos using Behavioral Observation Research Interactive Software (BORIS; Friard et al. 2016), counting the number of times the ants performed different types of behaviors, which were categorized as either submissive or aggressive based on the results of previous studies of ant behavior (Maák et al. 2019, Scharf et al. 2011; Table S1).

Statistical analyses

All the statistical analyses were performed in R Statistical Environment (R Core Team 2019). GLMs and GLMMs were carried out using the *glmer* function in the *lme4* package (Poisson and binomial distributions; Bates et al. 2015) and the *glmer.nb* and *glm.nb* functions in the *MASS* package (negative binomial distribution; Venables and Ripley 2002). For each of the GLMs, we performed Tukey pairwise post hoc comparisons between colonies using the *emmeans* function in the *emmeans* package (Russell 2019). We plotted our results using the *ggplot* function in the *ggplot2* package (Wickham 2016) and the *grid.arrange* function in the *gridExtra* package (Auguie

2017). Count data were analyzed using the Poisson distribution, except when the residuals were overdispersed. In those cases, we used the negative binomial distribution instead. Proportions were analyzed using the binomial distribution.

Testing differences in aggressiveness between species

We examined differences in how each species behaved during the two confrontation types (i.e., when numerically dominant versus numerically subordinate).

First, we examined the aggressive behavior displayed by the numerically subordinate ants during the two confrontation types using a generalized linear mixed-effects model (GLMM; Poisson distribution, fit by maximum likelihood). The dependent variable was the number of times the numerically subordinate ant displayed an aggressive behavior; confrontation type (HUMdom vs NEGdom) was a fixed factor; and colony identity was a random factor (to account for colony-related variability).

Second, we examined the aggressive behavior displayed by the numerically dominant ants during the two confrontation types. Not all the numerically dominant ants actively participated in the confrontations (% of confrontations with a given number of active numerically dominant ants: two—23.4%, three—29.8%, four—31.9%, and five—14.9%). Consequently, we analyzed that data using a GLMM with a binomial distribution: the dependent variable was the proportion of behaviors that were aggressive per active numerically dominant ant in each confrontation (calculated with the function *cbind*); confrontation type was a fixed factor; and colony identity was a random factor.

Testing differences in the behaviors and behavioral sequences of numerically subordinate ants

We examined differences in how each species behaved during the two types of confrontations (i.e., when numerically dominant versus numerically subordinate).

First, we examined differences in the behaviors (i.e., antennation, fleeing, mandible opening, biting, prolonged biting, and chemical attack) displayed by the numerically subordinate ant during the two

confrontation types using GLMMs (Poisson distribution, fit by maximum likelihood). The behavior thanatosis was excluded because of its rarity. We performed separate models for each behavior: the dependent variable was the number of times a behavior was performed; confrontation type was a fixed factor; and colony identity was a random factor.

Second, the same series of models was performed to examine the number of times the active numerically dominant ants displayed each behavior. However, in these models, the random factor was the number of the repetition nested in colony identity.

Finally, we looked at the relative frequency of different behavioral sequences and transitions for each species in confrontations where the species was numerically subordinate. Behavioral strings were extracted from BORIS, and Behatrix was used to build a flow diagram for each species.

Testing the relationship between aggressiveness and numerical dominance

We were interested in determining whether the aggressiveness displayed by each species depended on its numerical dominance during the confrontation. We examined such differences in aggressiveness using GLMs (negative binomial distribution); there was one GLM per species. The dependent variable was the number of times an ant displayed an aggressive behavior (either the numerically subordinate ant or the most aggressive of the five numerically dominant ants, depending on confrontation type), and confrontation type was a fixed factor.

Testing the relationships between aggressiveness, colony identity, and ant role

We were interested in determining whether aggressiveness could be influenced by other factors, such as colony identity and the confrontational role played by ants from a given colony.

First, we looked differences in aggressiveness among colonies of the same species using generalized linear models (GLMs; Poisson distribution, fit by maximum likelihood). We ran four models: one with data from numerically subordinate *L. humile* ants, one with data from numerically subordinate *L. neglectus* ants, one with data from numerically dominant *L. humile* ants, and one with data from numerically

dominant *L. neglectus* ants. The dependent variable was the number of times an ant displayed aggressive behavior, and colony identity was a fixed factor. In the models focused on the numerically dominant ants, we used the data for the most aggressive numerically dominant ant. To test for general differences among colonies, we carried out a Chi-squared test for each GLM using the *anova* function.

Second, we explored whether a colony's workers differed in their aggressiveness based on whether they were numerically subordinate or numerically dominant (GLM; negative binomial distribution). The dependent variable was the number of times the numerically subordinate ant or the most aggressive numerically dominant ant displayed aggressive behavior, and the ant's role (numerically subordinate or numerically dominant) was a fixed factor. We ran seven separate models: one for each colony of every ant species.

Testing the relationship between aggressiveness and species co-occurrence

We examined whether species co-occurrence affected aggressiveness. We were specifically interested in knowing whether ants from *L. humile* and *L. neglectus* colonies collected in Sant Cugat reacted differently to ants from colonies collected in areas where the species did not co-occur. We used four separate GLMs (Poisson distribution, fit by maximum likelihood) as described above (i.e., using data from numerically subordinate versus numerically dominant ants for each species). The dependent variable was the number of times the numerically subordinate ant or the most aggressive numerically dominant ant displayed aggressive behavior, and the presence/absence of species co-occurrence at a colony's area of origin was a fixed factor.

Results

Were there species-specific differences in the aggressiveness of the numerically subordinate ant?

The frequency of aggressive behavior by numerically subordinate ants differed significantly based on species. When each of the species was numerically

subordinate, *L. neglectus* more frequently displayed aggressive behavior than did *L. humile* ($z = -4.41$, $p < 0.001$; Fig. 2a). There was no species-specific difference in aggressiveness in numerically dominant ants ($z = -1.63$, $p = 0.08$; Fig. 2b).

The type of behavior displayed by numerically subordinate ants also differed based on species. When each species was numerically subordinate, *L. humile* engaged less frequently in behaviors such as antennation, biting, mandible opening, or chemical attacks than did *L. neglectus*; however, *L. humile*'s tendency to flee was significantly greater when it was the numerically subordinate ant (Fig. 3a). When each species was numerically dominant, *L. humile* less frequently engaged in fleeing, mandible opening, and chemical attacks than did *L. neglectus*; no significant differences were found for the other behavior categories (Fig. 3b).

Were there species-specific differences in behavioral sequences?

There were species-specific differences in the behavioral sequences displayed by the numerically subordinate ants. The most common behavioral sequence displayed by *L. neglectus* was more aggressive than the one displayed by *L. humile*. The Argentine ant was more inclined to start and finish confrontations with submissive behaviors whereas *L. neglectus* tended to keep up a high level of aggressiveness during the whole confrontation. In *L. humile*, the submissive behavior antennation was the first and most common behavior displayed by the ant. Antennation was followed by mandible opening in 39% of confrontations and by a second instance of antennation in 30% of confrontations. In *L. neglectus*, the repetition of antennation was much less common (19% of confrontations). Instead, antennation was followed by mandible opening and chemical attacks in 44% and 11% of confrontations, respectively. This sequence was slightly less common in *L. humile* (40% and 8% of confrontations, respectively). The Argentine ant tended to follow one instance of mandible opening with a second (40% of the confrontations) or with fleeing (16% of confrontations). In contrast, *L. neglectus* tended to start confrontations more aggressively (two sequential instances of mandible opening in 42% of confrontations). This behavioral sequence was generally followed up by either biting (15% of

confrontations) or a third instance of mandible opening (30% of the confrontations). In *L. humile*, the most aggressive behavior—chemical attack—was followed by a second chemical attack in just 14% of confrontations; in *L. neglectus*, this percentage was much higher: 23%. Finally, the most submissive behavior, thanatosis, was only performed by *L. humile*; it was never displayed by *L. neglectus* (Fig. 4a, b).

Were there species-specific differences in the aggressiveness of the numerically dominant ant?

There were species-specific differences in aggressiveness when ants were numerically dominant. Regardless of whether *L. humile* was numerically subordinate or dominant, its frequency of aggressive behaviors did not differ (Fig. 5a). In contrast, when *L. neglectus* was numerically subordinate, it more frequently displayed aggressive behaviors than when it was numerically dominant (Fig. 5b).

Were there relationships between aggressiveness, colony identity, and ant role?

Species displayed colony-specific differences in behavior both when numerically subordinate and numerically dominant (Chi-square test—*L. humile*: numerically subordinate: $\chi^2 = 42.44$, $df = 3$, $p < 0.0001$, numerically dominant: $\chi^2 = 118.98$, $df = 3$, $p < 0.0001$; *L. neglectus*: numerically subordinate: $\chi^2 = 38.77$, $df = 2$, $p < 0.0001$, numerically dominant: $\chi^2 = 62.02$, $df = 2$; $p < 0.0001$). However, the pairwise comparisons indicated that there were significant differences between some of the colonies. When *L. humile* was numerically subordinate, the Sant Cugat colony (representing the Catalan supercolony) was more aggressive than the Doñana and Girona colonies (both representing the Main supercolony) ($p \leq 0.003$ in both cases) but was not significantly different from the Seville colony (Fig. 6a, Table S2). By contrast, when *L. neglectus* was numerically subordinate, the Sant Cugat colony was less aggressive than the Gandía and Warsaw colonies ($p \leq 0.0001$ in both cases, Fig. 6b, Table S2). When *L. humile* was numerically dominant, the Sant Cugat colony behaved the least aggressively ($p < 0.001$, Fig. 6c, Table S2); when *L. neglectus* was numerically dominant, the opposite was true: the Sant Cugat

colony was the most aggressive ($p \leq 0.001$, Fig. 6d, Table S2).

Ants from different colonies behaved differently depending on their role in the confrontations (numerically subordinate vs. numerically dominant). In the case of *L. humile*, ants from the Sant Cugat colony were more aggressive when they were numerically dominant. By contrast, in *L. neglectus*, ants from the Sant Cugat colony were more aggressive when they were numerically subordinate (Fig. 7a, b, Table S3). In *L. humile*, ants from the Doñana colony were more aggressive when they were numerically subordinate (Fig. 7e, Table S3); however, the Seville and Girona colonies showed no such differences (Fig. 7c, g, Table S3). In *L. neglectus*, ants from Gandía, Warsaw, and Sant Cugat colonies were more aggressive when they were numerically subordinate (Fig. 7d, f, Table S3).

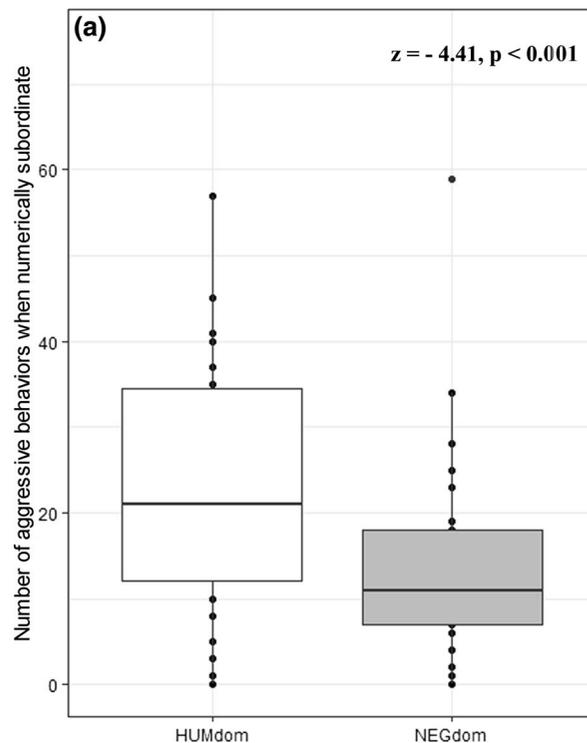
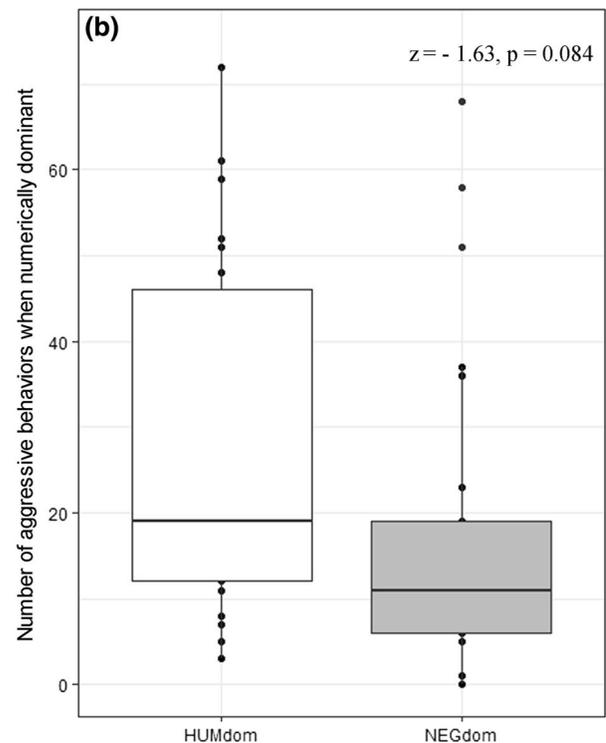


Fig. 2 Frequency of aggressive behaviors displayed by **a** numerically subordinate and **b** numerically dominant ants during the two confrontation types (HUMdom: *L. humile* was numerically dominant; NEGdom: *L. neglectus* was numerically

Fig. 3 Frequency of the different behavior types displayed by **a** the numerically subordinate ant ($n = 24$) and **b** the numerically dominant ants ($n = 24$) during the two confrontation types (HUMdom = *L. humile* was numerically dominant; NEGdom = *L. neglectus* was numerically dominant). The statistical results are at the top of each graph; they are in bold when significant. The boxplots show the median, upper and lower quartiles, minimum and maximum, and any outliers

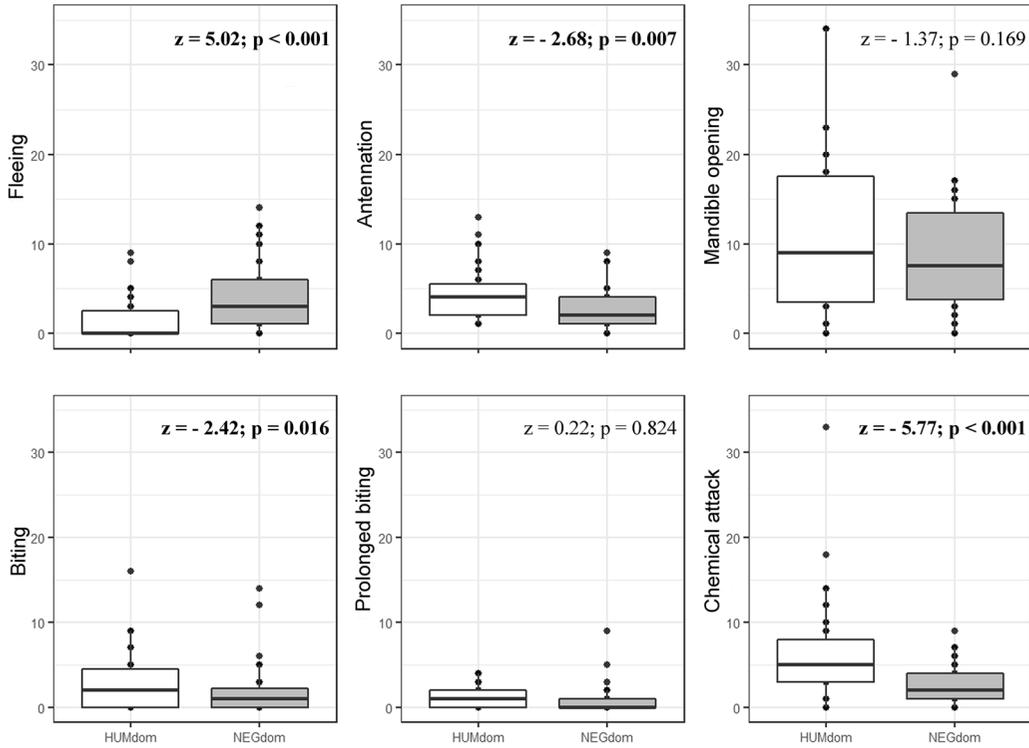
Was there a relationship between aggressiveness and species co-occurrence?

When we specifically examined how species co-occurrence affected aggressiveness, we found that numerically dominant *L. humile* ants from Sant Cugat were significantly less aggressive toward numerically subordinate *L. neglectus* ants from Sant Cugat versus from other colonies (Fig. 8c); however, no such difference was found when *L. humile* was numerically subordinate (Fig. 8a). Moreover, numerically subordinate *L. neglectus* ants from Sant Cugat behaved

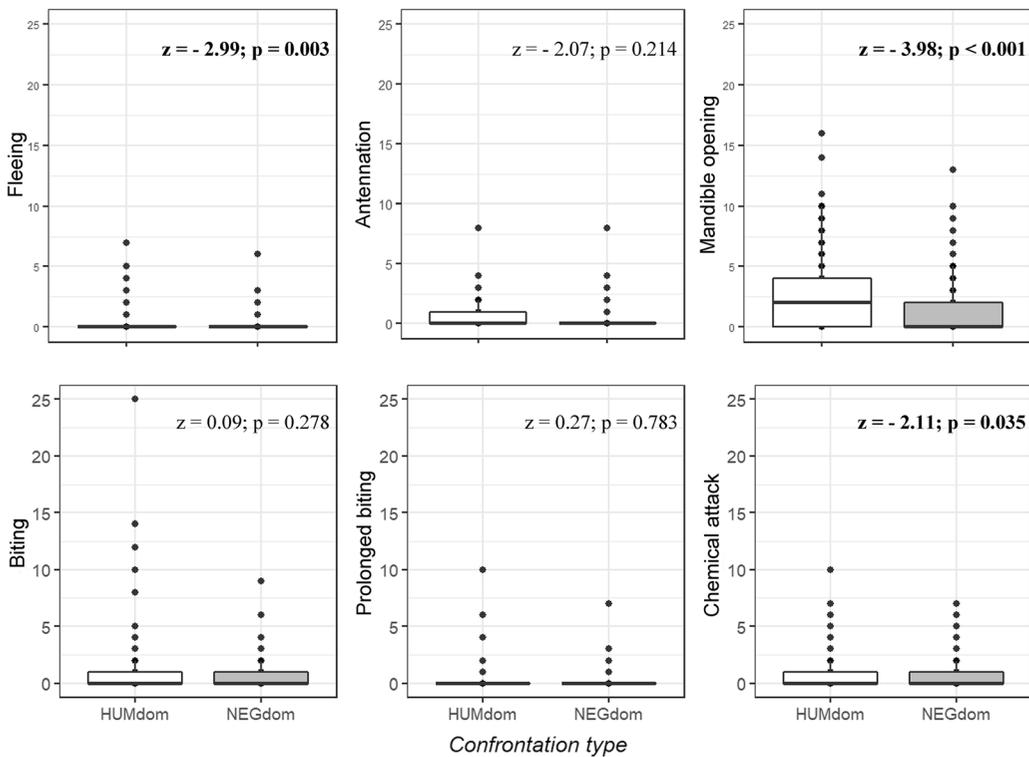


dominant; $n = 24$). The statistical results are at the top of each graph; they are in bold when significant. The boxplots show the median, upper and lower quartiles, minimum and maximum, and any outliers

(a) Frequency of behaviors of the numerically subordinate ant



(b) Frequency of behaviors of the numerically dominant ant



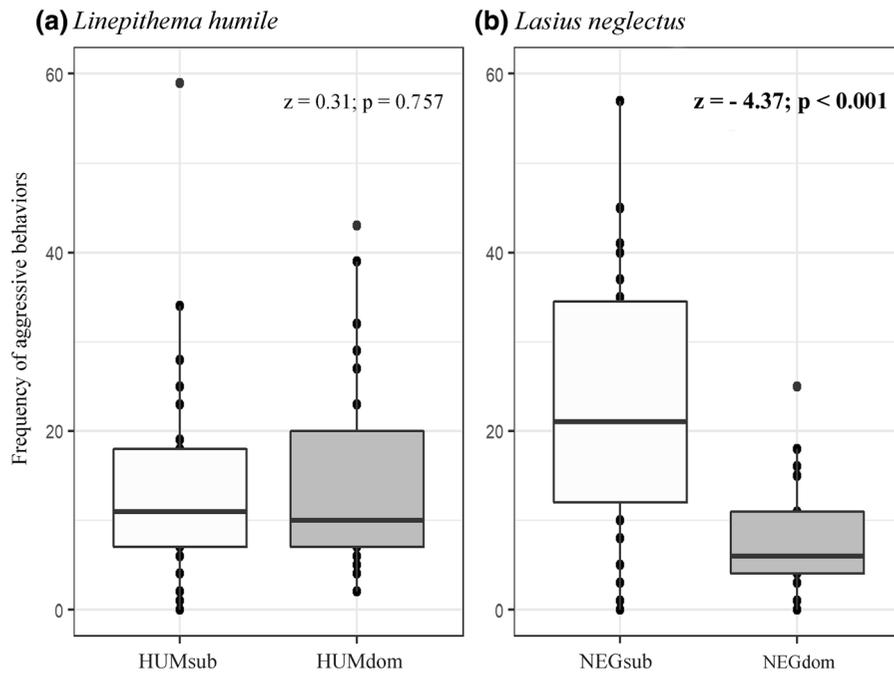


Fig. 5 Frequency of aggressive behaviors displayed by **a** *L. humile* and **b** *L. neglectus* when numerically subordinate versus numerically dominant ($n = 24$ trials for each confrontation type for each species). The statistical results are at the top of each

continent's native biodiversity (Gómez et al. 2003; Gómez and Oliveras 2003; Blancafort and Gómez 2006; Angulo et al. 2007; Nagy et al. 2009; Abril and Gómez 2011). The two species have similar ecologies and thus their ranges could potentially overlap; they might even end up co-occurring in many areas, as they already do in Sant Cugat, Spain. *Linepithema humile* arrived in Europe before *L. neglectus*, but the latter dominates the former in confrontations involving equal numbers of ants (Bertelsmeier et al. 2015c). Consequently, we wished to test the effect of numerical dominance on their interactions. Our results show that *L. neglectus* remained behaviorally dominant and highly aggressive even when it was numerically subordinate to *L. humile*. However, in interactions involving ants from Sant Cugat, where the species co-occur, *L. humile* but not *L. neglectus*, seemed to employ the dear-enemy strategy, reducing in part the level of aggressiveness during confrontations. Thus, our study describes how *L. humile* and *L. neglectus* can modulate their behaviors, and the background mechanisms that can facilitate the co-occurrence of these two highly aggressive invasive species.

graph; they are in bold when significant. The boxplots show the median, upper and lower quartiles, minimum and maximum, and any outliers

Aggressiveness and numerical dominance: the Bourgeois strategy

Invasive ants share a series of ecological and biological traits, which are often collectively called the “invasive syndrome” (Passera 1994). These traits facilitate their success during invasions (Bertelsmeier et al. 2017; Fournier et al. 2017). However, these similarities may also result in invasive species competing for the same resources when they co-occur, with competitive displacement as the final outcome. Indeed, several studies have observed the invasion treadmill phenomenon, where two invasive ant species initially co-occur but later one displaces the other (Thomas and Reid 2007; LeBrun et al. 2007; Krushelnycky and Gillespie 2010; Spicer Rice and Silverman 2013). However, the invasive syndrome does not imply that all invasive ant species display equal levels of aggressiveness; instead, they are organized into a dominance hierarchy (Bertelsmeier et al. 2015a). However, the linear hierarchy established by Bertelsmeier et al. (2015a) was based on species confrontations involving similar numbers of competitors. The results may have differed if competitor number

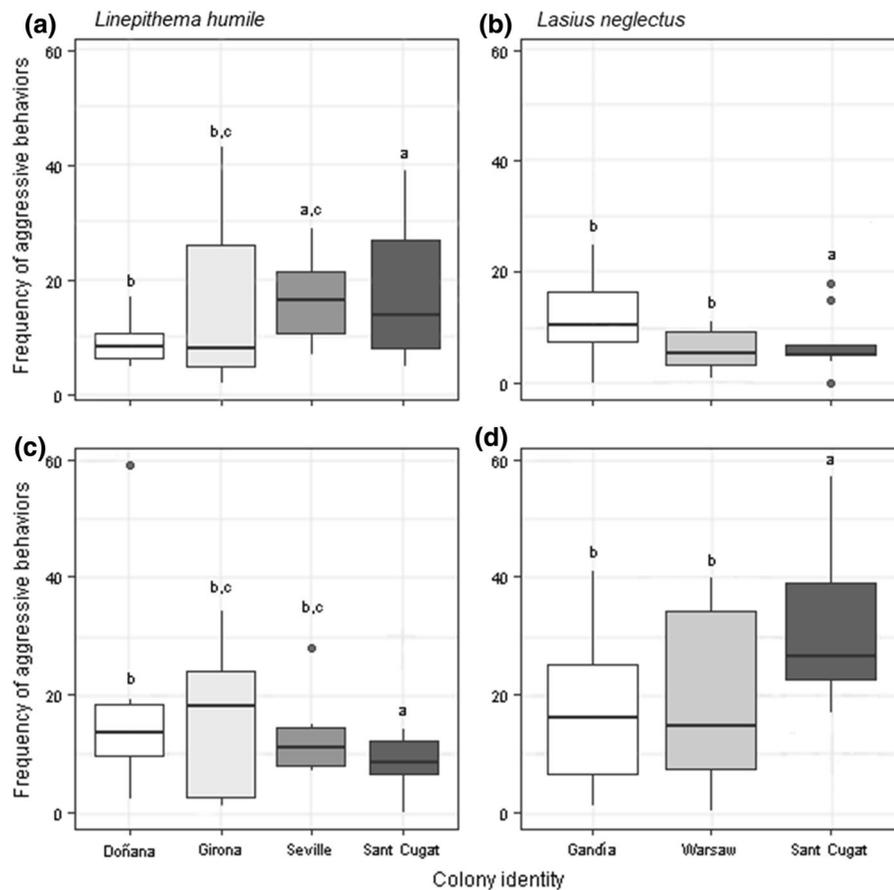


Fig. 6 Frequency of aggressive behaviors displayed by ants from different colonies of numerically subordinate **a** *L. humile* and **b** *L. neglectus* and of numerically dominant **c** *L. humile* and **d** *L. neglectus* ($n = 12$ trials for each confrontation type for each colony of each species). In **c**, **d** the data came from the most

aggressive of the numerically dominant ants. The boxplots show the median, upper and lower quartiles, the minimum and maximum, and any outliers; boxes with the same letter were not statistically different

had been unequal. In our study, we observed that *L. neglectus* displayed a consistent level of aggressiveness: ants from all the colonies were always more aggressive in confrontations with *L. humile* when they were numerically subordinate. This pattern was seen in both the higher number of aggressive interactions and the type of behaviors exhibited: *L. neglectus* employed more aggressive behaviors, such as biting or chemical attacks, while *L. humile* mostly performed submissive behaviors, such as fleeing or thanatosis. These results concur with those of Cremer et al. (2006), who showed that the invasive *L. neglectus* displayed a higher frequency of aggressive behaviors, notably biting and chemical attacks, than did native *Lasius* species.

However, aggressiveness is defined by more than just the frequency of aggressive behaviors; it is also the sequence in which behaviors are performed. For example, if an ant progressively uses more aggressive behavior, a confrontation can become more violent. In our study, *L. neglectus* was not only more frequently aggressive, it also employed increasingly aggressive behavioral types. Like Bertelsmeier et al. (2015a, 2016), we found that *L. neglectus* has better competitive skills than *L. humile*, even if the latter displays numerical dominance. Indeed, when arriving in an area already colonized by *L. humile*, *L. neglectus* can utilize this advantage to better compete for resources, allowing it to occupy a higher position in the dominance hierarchy (Bertelsmeier et al. 2015a, 2016).

It is also important to note that competition strategies may be flexible and can shift to increase the probability of success (Suárez et al. 1999). The particular strategy employed during interspecific conflicts depends on different factors, such as competitor abundance and aggressiveness; environmental conditions; social conditions; and the duration of the interaction (DiGirolamo and Fox 2006; Frizzi et al. 2017; Abril and Gómez 2009; Blight et al. 2010; Kleineidam et al. 2017). Indeed, in ant species, competitor abundance and aggressiveness play a key role in interspecific interactions and may vary in order to boost competitive ability. For example, it has been shown that *L. humile* and some native Mediterranean ant species altered their behavior based on the number of conspecifics present on baits, thus adopting the Bourgeois strategy (Carpintero and Reyes-López 2008). Indeed, native and exotic species all around the world use the Bourgeois strategy to expulse other species and monopolize resources using aggressive behaviors (Holway and Suárez 1999; Drescher et al. 2010). Here, we found that *L. humile* used the Bourgeois strategy in confrontations with *L. neglectus*. When *L. humile* was numerically subordinate, it displayed more submissive behaviors, such as thanatosis or fleeing. However, when it was numerically dominant, it displayed a greater frequency of aggressive behaviors. Interestingly, Blight et al. (2010) also observed that *L. humile* workers engaged in thanatosis when they were losing confrontations. This submissive behavior has also been observed in *Plagiolepis pygmaea* in confrontations with *L. humile*, and it may greatly contribute to the co-occurrence of these two species in the Mediterranean (Abril and Gómez 2009).

Aggressiveness and co-occurrence: the dear-enemy hypothesis

Behavioral interactions among ant species are affected not only by behavioral dominance or numerical dominance but also by colony personality (Carere et al. 2018; Blight et al. 2017). Indeed, behavior and competitive ability can vary among colonies of the same species. For example, in *L. humile* in Europe, ants from the Main supercolony were found to be more aggressive and to more readily explore novel environments than ants from the Catalan supercolony (Abril and Gómez 2011; Blight et al. 2017). The results of our study support these previous findings

(Abril and Gómez 2011) because, when *L. humile* was numerically subordinate, ants from the Catalan supercolony (Sant Cugat) were less aggressive than ants from the Main supercolony (Doñana and Girona).

The behavioral plasticity displayed by *L. humile* in response to local conditions probably facilitates invasion success (Holway and Suárez 1999; Sagata and Lester 2009) and can therefore shape the outcome of situations in which *L. humile* co-occurs with another invasive species, such as *L. neglectus*. When we staged confrontations between ants taken from an area where *L. humile* and *L. neglectus* co-occur (Sant Cugat), we observed that numerically dominant *L. humile* ants were less aggressive toward numerically subordinate *L. neglectus* ants than when the confrontations involved ants from areas where there is no species co-occurrence. We found similar results for *L. neglectus*: numerically subordinate *L. neglectus* ants were less aggressive toward familiar versus foreign numerically dominant *L. humile* ants. This finding suggests the dear-enemy strategy may facilitate the co-occurrence of these two invasive ant species in this particular area. Our results also support the idea that *L. humile* can use its behavioral plasticity to persist in the presence of *L. neglectus*, despite the latter's greater aggressiveness and better competitive skills. In their natural range, the coevolution of the different species lead to some of them to evolve a subordinate strategy towards the dominant species, an strategy also displayed in invaded areas as a result of their life-history tradeoff (Morrison 1996). For example, part of the invasion success of *L. humile* could be related with escaping from coevolved natural enemies (Holway and Suárez 1999), as it is the case with *L. neglectus* in San Cugat. This shift in behavior could be the underlying cause of the dear-enemy phenomenon that was observed. This phenomenon (Fisher 1954) is common in social insects, and it has been previously described in other ant species (Heinze et al. 1996; Boulay et al. 2007; Grangier et al. 2007; Dimarco et al. 2010). Furthermore, *L. humile* has also been found to co-occur with other invasive species such as *Cardiocondyla mauritanica* (Gómez and Espadaler 2005) in Balearic Islands, and with *Cardiocondyla obscurior* and *Tetramorium caldarium* in Alicante and Andalusia (Trigos-Peral and Reyes-López 2016; Trigos-Peral et al. 2020). Consequently, it is possible that the above mentioned behavioral plasticity of *L. humile* may facilitate its co-occurrence with other invasive species.

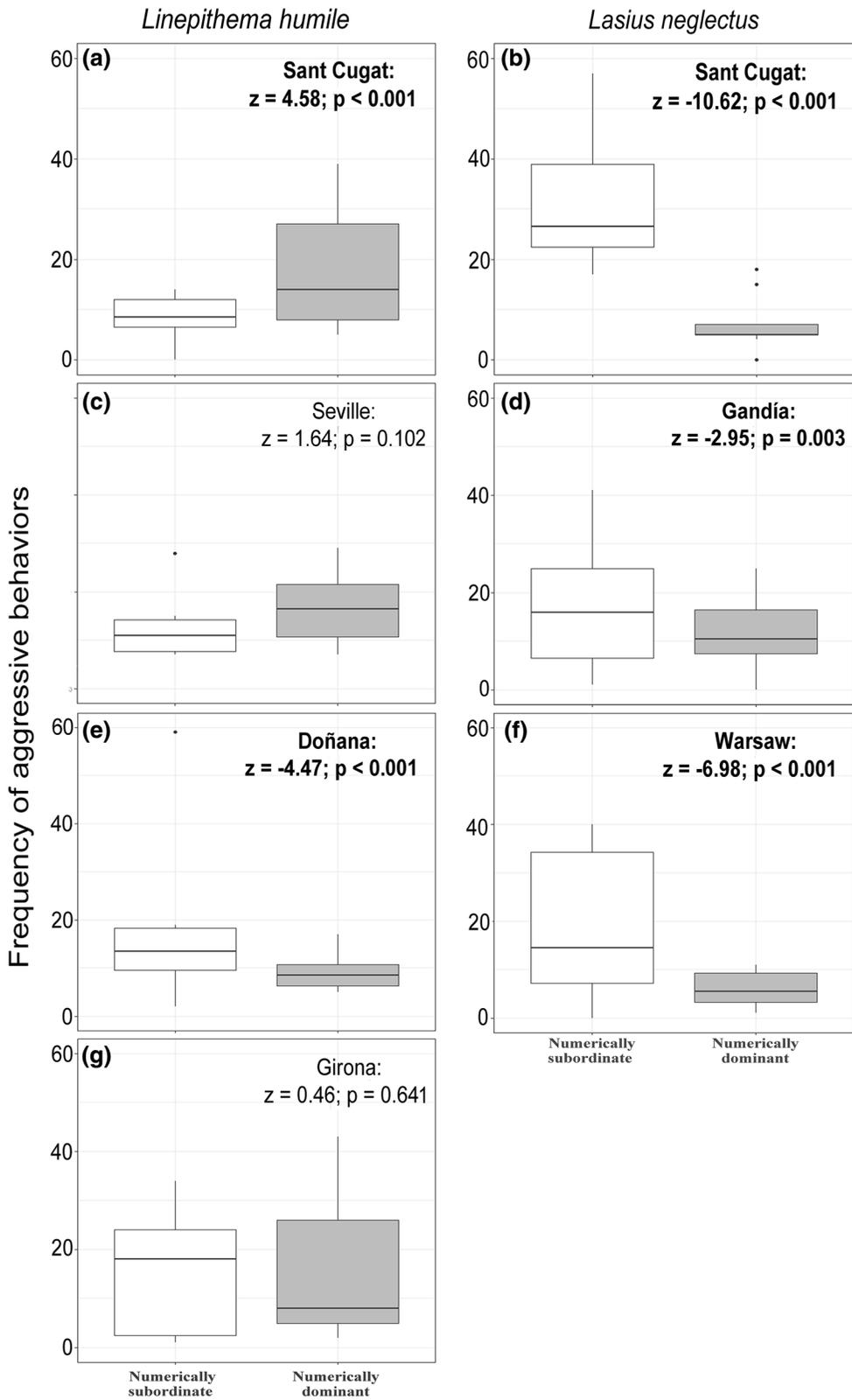


Fig. 7 Frequency of aggressive behaviors displayed by numerically subordinate versus numerically dominant ants from different colonies of *L. humile* (a, c, e, g) and *L. neglectus* (b, d, f) (n = 12 trials for each confrontation type for each colony of each species). The statistical results are at the top of each graph; they are in bold when significant. The boxplots show the median, upper and lower quartiles, minimum and maximum, and any outliers

Study limitations and implications

The competitive success of a given species is often determined by the suitability of local environmental conditions, species abundance, and species aggressiveness (Buczowski and Bennett 2007; Blight et al. 2010; Bertelsmeier et al. 2015a, b; Frizzi et al. 2017). In our study, we assumed that environmental

conditions played a secondary role since *L. humile* and *L. neglectus* share ecological similarities. However, because they come from different geographical areas (South America and the Anatolian Peninsula, respectively), local-scale environmental conditions in their invasive ranges might differentially affect their behavioral responses during confrontations in the field.

We also assumed that species abundance was correlated with species time of arrival. The first invasive species to arrive within a new area can become numerically dominant, increasing its likelihood of outcompeting a later-arriving species (Bang et al. 2017). Previous work has shown that only some cryptic native ant species remained in areas that *L. humile* had successfully invaded (and where its nests were larger and more numerous) while the native ant

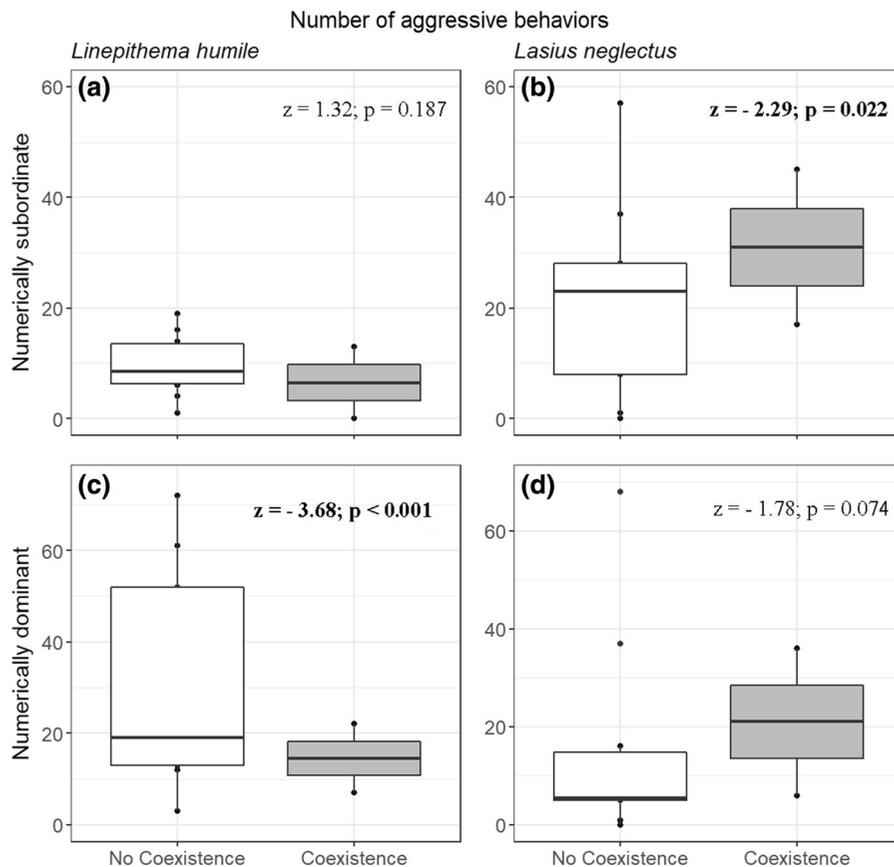


Fig. 8 Frequency of aggressive behaviors displayed by numerically subordinate a *L. humile* and b *L. neglectus* and numerically dominant c *L. humile* and d *L. neglectus* in confrontations involving colonies from areas where the species did and did not co-occur (n = 12 trials for each confrontation

type for each species in each type of area). The statistical results are at the top of each graph; they are in bold when significant. The boxplots show the median, upper and lower quartiles, minimum and maximum, and any outliers

community still co-occurred with *L. humile* near the invasion front (Diaz et al. 2014).

Our study did not address certain other factors that might influence species behavior and that might be involved in the establishment and success of invasive species. For example, ants could not fully flee from the confrontations staged in our experimental trials. Since natural habitat complexity could strongly influence species' responses and promote co-occurrence (Gibb and Parr 2010; Sarty et al. 2006), our trials could have yielded very different results if carried out in the field; indeed, the dear-enemy effect might have been stronger.

That said, the controlled conditions of laboratory experiments make it possible to test whether species are specifically responding to the applied treatments (e.g., by excluding environmental variability). Laboratory experiments are especially useful in the field of invasion biology because they can be used as tools for predicting the possible outcomes of as-yet infrequent scenarios that are expected to become more common, such as the co-occurrence of the two invasive species *L. humile* and *L. neglectus*.

Additionally, temperature has been shown to be a key factor that mediates interspecific competition. Frizzi et al. (2017) found that the competitive ability of *L. neglectus* was lower at higher temperatures. This fact may give a competitive advantage to *L. humile* in the areas where these two species co-occur, given that *L. humile* is slightly better at tolerating higher temperatures and drier conditions (Jumbam et al. 2008). Global warming seems to be greatly enhancing habitat suitability for invasive species by increasing resource availability, decreasing the resistance of native communities to the establishment of invasive species, and giving invasive species a competitive advantage over already established non-native species (Diez et al. 2012). Thus, climate change may allow *L. humile* and *L. neglectus* to spread to colder parts of Europe (Roura-Pascual et al. 2004; Bertelsmeier and Courchamp 2014; Stukalyuk 2017). Indeed, current models exploring the distributions of invasive ant species under climate change conditions have predicted that these species will continue spreading as average global temperature increases (d'Ettorre and Lenoir 2010). Thus, interactions between temperature and ant numerical dominance merit further research.

Our study is the first to show how behavioral plasticity in *L. humile* may greatly facilitate the

species' co-occurrence with other highly aggressive invasive species, such as *L. neglectus*. Because of global warming, the invasion of the same habitat by multiple species is a reality, and both the bourgeois and dear-enemy strategies may facilitate species establishment. Our results also highlight the importance of conducting future research exploring the potential of the invasive species to co-occur in base to their morphological traits, aggressiveness and behavioral plasticity; such work can help reveal how both factors affect native communities and environmental conservation.

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Table S1. Behaviors observed during the confrontations and their descriptions.

Behaviors			
Submissive		Aggressive	
Behavior	Description	Behavior	Description
Antennation	Touching the opponent with one's antennae	Mandible opening	Opening one's mandibles in the opponent's presence
Fleeing	Running away upon perceiving the presence of the opponent	Biting	Rapidly biting the opponent
Thanatosis	Feigning death to escape from the opponent	Prolonged biting	Biting the opponent for longer than 2 seconds
		Chemical attack	Spraying acid at the opponent

Table S2. Differences between colonies in the frequency of aggressive behavior for numerically subordinate and numerically dominant ants of *L. humile* and *L. neglectus*. The statistics shown are from Tukey pairwise post-hoc comparisons (n = 24 for each); they are in bold when significant.

<i>Numerically subordinate ant</i>					
<i>Linepithema humile</i>			<i>Lasius neglectus</i>		
Colonies	z	p	Colonies	z	p
Sant Cugat vs Doñana	4.97	< 0.001	Sant Cugat vs Gandía	5.47	< 0.001
Sant Cugat vs Girona	3.48	0.003	Sant Cugat vs Warsaw	5.02	< 0.001
Sant Cugat vs Seville	2.53	0.055	Warsaw vs Gandía	0.72	0.751
Doñana vs Girona	1.82	0.265			
Doñana vs Seville	2.63	0.042			
Girona vs Seville	0.94	0.782			
<i>Numerically dominant ants</i>					
Doñana vs Sant Cugat	4.75	< 0.0001	Sant Cugat vs Gandía	3.74	< 0.001
Girona vs Sant Cugat	2.75	0.030	Sant Cugat vs Warsaw	4.08	< 0.001
Seville vs Sant Cugat	4.37	0.0001	Warsaw vs Gandía	0.26	0.962
Doñana vs Seville	0.44	0.968			
Doñana vs Girona	2.26	0.109			
Girona vs Seville	-1.85	0.257			

*Significant results in bold

Table S3. Differences in the frequency of aggressive behaviors displayed by ants from different colonies of *L. humile* and *L. neglectus* when they were numerically subordinate versus numerically dominant. The statistics shown are from Tukey pairwise post-hoc comparisons (n = 12 for each); they are in bold when significant.

Colony identity	<i>Linepithema humile</i>	
	<i>z</i>	<i>P</i>
Sant Cugat	4.58	< 0.001
Seville	1.64	0.102
Doñana	- 4.47	< 0.001
Girona	0.46	0.641
Colony identity	<i>Lasius neglectus</i>	
	<i>z</i>	<i>P</i>
Sant Cugat	- 10.62	< 0.001
Gandía	- 2.95	0.003
Warsaw	- 6.98	< 0.001