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Not dear neighbours: Antennation and jerking, but not aggression, correlate with genetic relatedness and spatial distance in the ant *Lasius niger*

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Abstract

1. Neighbour–stranger response differences (NSRDs) are when individuals are either more aggressive ("Nasty Neighbour") or less aggressive ("Dear Enemy" or "Dear Neighbour") to direct neighbours than to other competitors perceived as "strangers" by the residents. Such effects are often reported in ants which, being fixed-location central-place foragers, may compete directly with their neighbours for resources or raid each other for brood. Overlayed onto this are potential spatial distance and relatedness effects on aggression, which are often not differentiated from NSRDs.
2. The literature on NSRDs and distance effects in ants does not reveal a systematic pattern across all ants due to their diversity of life histories, requiring each species to be evaluated individually. *Lasius niger* is a common Eurasian ant species, which can form very dense populations of colonies and shows pronounced nestmate recognition, so may be expected to show NSRDs.
3. Here, we take advantage of a semi-regular colony array to examine the effect of spatial distance and relatedness on aggression and probe for NSRDs.
4. Overt aggression does not vary with relatedness or spatial distance, and there is no evidence that direct neighbours represent a special case in terms of aggression. However, antennation and jerking decrease between less related and more spatially distant pairs, but are almost completely absent from allospecific interactions.
5. We tentatively propose that antennation and jerking together represent a 'negotiation' phase, which may either precede or reduce the need for overt aggression. While a Nasty Neighbour effect might occur, a Dear Neighbour effect is unlikely in this species, and overall NSRDs do not play a large role in the ecology of this species. More broadly, this work highlights the importance of considering non-overtly aggressive responses when studying NSRDs.

KEYWORDS
aggression, Dear Neighbour effect, Formicidae, inter-colony aggression, neighbour–stranger discrimination

INTRODUCTION

Competition for resources between animals may result in both inter- and intraspecific aggressions. However, since aggression can be costly, aggressive responses are modulated depending on the potential costs and gains each actor faces. For species that protect long-term territories or those that use their territories for multiple purposes, a major proximate aspect modulating aggression is whether the interaction is between direct neighbours or unknown individuals—so called neighbour–stranger response differences (NSRDs) (reviewed in Christensen & Radford, 2018; Temeles, 1994). Such NSRDs can occur at both the intra- (e.g., Yagound et al., 2017) and interspecific (e.g., Tanner & Adler, 2009) levels. Compared with interactions involving stranger (i.e., non-neighbours) individuals, direct neighbours may either be responded to with reduced aggression, termed the *Dear Neighbour Effect*, or with increased aggression, termed the *Nasty Neighbour Effect*. Whether a Dear Neighbour or Nasty Neighbour effect is found relates to the ecology and environment of the studied system.

A Dear Neighbour effect (senso reviewed in Temeles, 1994, also termed the "Dear Enemy" effect) is often to be expected, because the behaviour of established neighbours is better known. They are, thus, more predictable (Amorim et al., 2022; Getty, 1987) and are less likely to compete for resources, since they have their own (Jaeger, 1981; Switzer et al., 2001). However, in some situations, direct neighbours represent a greater threat, and thus, a Nasty Neighbour response is expected (Müller & Manser, 2007). This might occur in situations where neighbours can usurp territory and gain by this (e.g., by access to more, better or more reliable feeding sites), or when established neighbours are more powerful than non-neighbours. For example, banded mongooses often usurp the territory of their neighbours (Cant et al., 2002) and pose a bigger threat than roving bands of dispersing splinters (Cant et al., 2001). They, thus, do not show a Dear Neighbour effect, but rather a Nasty Neighbour effect, where aggression is higher towards direct neighbours (Müller & Manser, 2007). Similarly, less resource-rich areas lead to an increasing competition for food, and thus aggressiveness in *Formica aquilonia* (Sorvari & Hakkarainen, 2004). In essence, whether NSRDs occur, their direction and their strength depend primarily on the difference in threat levels between neighbours and strangers. This is also influenced by how much the focal animal stands to lose. The strength and direction of NSRDs strongly depend on species and breeding stage (Werba et al., 2022).

While NSRDs were initially studied in solitary species, recently, a greater focus has been on the response of social species (reviewed in Christensen & Radford, 2018). The NSRDs of social animals can differ greatly from non-social animals for two reasons. First, inter-individual differences in a group, in terms of personality, status and resources (Beehner & Kitchen, 2007; Desjardins et al., 2008), mean that some individuals in a group are more likely to respond to territorial incursion than others—depending on what they stand to gain or lose (Mares et al., 2011; York et al., 2019). For example, dominant male meerkats respond more strongly to cues of male invaders than females or non-dominants, because they risk losing their position as the main group breeder (Mares et al., 2011). Second, social groups must respond collectively to incursions, both to better handle the threat and to improve information transfer (Graw & Manser, 2007). Finally, cooperative, not just competitive, behaviour can vary between neighbours and strangers. For example, tree ants *Oecophylla smaragdina* can rescue conspecifics trapped in spiderwebs. They show this behaviour not just to nestmates but also to ants from neighbouring colonies, but not from distant colonies (Uy et al., 2019). Similarly, great tits are more likely to assist conspecifics in nest defence if they are very familiar with the conspecifics (Grabowska-Zhang et al., 2012).

Eusocial insects are a special case for NSRDs. Like other social animals, they must often mount collective responses to incursions (Bradshaw et al., 1975; Hölldobler, 1981; Whitehouse & Jaffe, 1996). However, as in most social insect colonies reproduction is performed overwhelmingly by the reproductive caste (Wilson, 2000), the motivations of all individuals are usually aligned in foraging and defence contexts, and the colony is in many respects effectively one solitary superorganism (Boomsma & Gawne, 2018). Competition for territory and resources, including tournaments and raids, are repeatedly reported in ants (Czechowski, 1984; Hölldobler, 1976; Pollock & Rissing, 1989), making them especially interesting for the study of NSRDs. However, no clear pattern of NSRDs can be seen for ants, with several studies reporting Dear Neighbour effects, others reporting Nasty Neighbour effects, and yet others reporting complex interactions or no effect (Table 1). This is not surprising, given that the presence, direction and strength of NSRDs depend on the specific ecology of the species: whether conspecifics are the largest competitor (Fogo et al., 2019), whether the species maintains distinct territories (Boulay et al., 2007), whether the distance between these territories is small or large (Zorzal et al., 2021) and whether competition over rare resources or raiding is common (Tumulty & Bee, 2021). NSRDs in ants are also strongly influenced by seasonal effects (Ichinose, 1991; Katzerke et al., 2006; Thurin & Aron, 2008). We note, however, that several studies do not differentiate spatial distance from neighbour–non-neighbour differences. This is an important distinction, as apparent NSRDs may be a result of spatial distance or genetic differences: in ants and other eusocial insects, nestmate recognition, and thus aggression to incursions by non-nestmates, is driven by colony-specific cuticular hydrocarbon (CHC) profiles (reviewed in van Zweden & d'Ettorre, 2010). CHC profiles may be influenced by genetic and environmental differences. Thus, overlaid on top of NSRDs, aggression is expected to increase with both relatedness and spatial distance since spatial distance can correlate with environmental differences. All such correlations have been reported (genetic: Aksoy & Çamlitepe, 2018; Saar et al., 2014, spatial distance: Jutsum et al., 1979; Sanada-Morimura et al., 2003, substrate and forage: Heinze et al., 1996; Jutsum et al., 1979; Stuart, 1987), but importantly, none of these measures are universally predictive of aggression. As such, to understand the ecology of a specific species, direct experimentation is required.

Here, we study the role of being close neighbours, spatial distance, and relatedness on aggression in the ant *Lasius niger*. We take advantage of a regularly spaced array of *L. niger* nests, allowing an

TABLE 1 Literature summary of neighbour–stranger response differences in ants.

Species	Details	Reference
Nasty neighbour effect (higher aggression to neighbours)		
Pristomyrmex pungens	Aggression decreased with spatial distance. Experiential exposure to a colony subsequently increased aggression towards that colony.	(Sanada-Morimura et al., 2003)
Linepithema humile	This ant species forms large supercolonies. Workers from different supercolonies are always aggressive to each other, but aggression is highest between ants from neighbouring nests, implying experience drives increased aggression. Relatedness and genetic similarity do not predict aggression.	(Thomas et al., 2007)
Oecophylla smaragdina	Higher proportion of non-nestmates recognised as such from neighbouring colonies. Once recognised as non-nestmates, higher aggression towards ants from neighbouring colonies.	(Newey et al., 2010)
Formica pratensis	Higher aggression between direct neighbours than between 'second neighbours' and non-neighbours.	(Benedek & Kobori, 2014)
Cataglyphis niger	Aggression between colonies from different populations lower than between colonies from the same population. Genetic and CHC profile differences were larger between than within populations.	(Saar et al., 2014)
Crematogaster scutellaris	Aggression decreases with increasing spatial and CHC differences. CHC profiles do not covary with relatedness.	(Frizzi et al., 2015)
Azteca muelleri	Higher aggression between non-sympatric ant pairs than sympatric ant pairs. No relationship between aggression and overall CHC similarity, but signs of higher methylated alkane similarity linked to higher aggression.	(Zorzal et al., 2021)
Dear neighbour effect (lower aggression to neighbours)		
Acromyrmex octospinosus	Aggression increases with distance between nests. Laboratory studies suggest both forage type and endogenous (presumably genetic) differences drive aggression.	(Jutsum et al., 1979)
Temnothorax curvispinosus	Lower aggression between colonies collected in the same area. After extended housing in the lab, aggression between stranger colonies decreased.	(Stuart, 1987)
Temnothorax nylanderi	Both increasing spatial distance and nesting material differences increased intra-specific aggression. Spatial distances between colonies tested ranged from 0 – >3 meters. Paired colonies transferred into different nesting material increased aggression, pairs with matching nesting materials did not.	(Heinze et al., 1996)
Iridomyrmex purpureus	Experiments using live ants and CHC extracts both find increasing aggression with spatial distance. Aggression increased in areas with a high density of conspecific nests.	(Thomas et al., 1999)
Formica exsecta	Aggression increases with spatial distance, but only in spring, not summer or autumn. Aggression was not correlated with genetic distance or intranest relatedness.	(Katzerke et al., 2006)
Acromyrmex lobicornis	Aggression increases with spatial distance. Genetic distance did not correlate with spatial distance.	(Dimarco et al., 2010)
Formica pratensis	Aggression increases with spatial and/or genetic distance, which themselves covary. Moving ants to the laboratory removes this effect, implying either context dependence or an effect of nesting substrate on aggression.	(Aksoy & Çamlitepe, 2018)
Oecophylla smaragdina	Rescue behaviour is directed towards nestmates and neighbours, but not conspecifics from distant colonies.	(Uy et al., 2019)
Both, none, or "it's complicated"		
Pogonomyrmex barbatus	Encounters with neighbours on a foraging trail reduce foraging more than encounters with non-neighbours. This stronger response may reduce costly aggression.	(Gordon, 1989)
Pheidole tucsonica & *P. gilvescens*	*P. tucsonica* show higher aggression towards conspecific from distant areas. When only ants from within a local area were tested, no effect of distance on aggression was found. *P. gilvescens* ants show no effect of distance on aggression.	(Langen et al., 2000)
Iridomyrmex purpureus	Higher aggression between ants from adjoining territories than ants from non-adjoining territories, but within these groupings more aggression between more distant colonies. No influence of genetic similarity on aggression. No evidence of experience modulating aggression.	(van Wilgenburg, 2007)

(Continues)

TABLE 1 (Continued)

Species	Details	Reference
Camponotus cruentatus	No difference in aggression between sympatric neighbours, sympatric non-neighbours, or allopatric pairs.	(Boulay et al., 2007)
Liometopum microcephalum	Arboreal ants with limited nest sites, so high competition. Compared aggression to direct neighbours, colonies in shared tree patch, and colonies in a distant tree path. Slightly higher aggression to neighbouring nest than stranger nest in shared patch. More pronounced increase in aggression to ants from distant patches	(Keresztes et al., 2020)

Note: This table includes only studies of intraspecific aggression.

unusually controlled spatial distance between the colonies. This model species is of particular relevance to study NSRDs for several reasons. *L. niger* is an extremely widespread species, dominating especially anthropogenically disturbed and open habitats throughout the Palearctic (Arnan et al., 2017; Guenard et al., 2017; Janicki et al., 2016). It is strictly monogynous, with occasional polyandrous colonies (Boomsma & Van Der Have, 1998; Fjerdingstad et al., 2002; van der Have et al., 1988). Dispersal is by alate gynes (winged female sexuals), which after mating found colonies claustrally (Fjerdingstad et al., 2003). *L. niger* is aggressive and, thus, may play an important role in resisting the spread of invasive ants into an ecosystem (Cordonnier et al., 2020). Furthermore, it is an important model organism for studying a wide variety of ecological and behavioural topics, such as defence and nestmate recognition (Devigne et al., 2004; Lenoir et al., 2009), biotic interactions (Blanchard et al., 2021; Detrain et al., 2010; Verheggen et al., 2012), collective behaviour (Beckers et al., 1990; Czaczkes et al., 2016; Dussutour et al., 2005) and cognition (De Agrò et al., 2022; Oberhauser et al., 2020; Poissonnier et al., 2023). Nest density can be high in suitable habitats, reaching almost 400 colonies per hectare (Boomsma et al., 1982) (see also Figure 1b). Colonies compete for nesting and food resources, and raid each other for brood, with tournaments being reported (Czechowski, 1984). A reduction in the production of sexuals with increasing nest density (Boomsma et al., 1982) and increasing production of sexuals if neighbouring colonies are poisoned (Pontin, 1961) implies that intraspecific competition is an important ecological factor for this species. Colonies maintain distinct territories that they defend from conspecifics (Czechowski, 1984; Devigne & Detrain, 2002; Fourcassié et al., 2012). It seems likely that direct neighbours are the greatest source of competition for resources and the greatest raiding threat. One might, thus, expect pronounced NSRDs in this species.

MATERIALS AND METHODS

Biological material

The ants used for the experiments came from 11 *L. niger* colonies sampled between 12 and 24 August 2021 in Regensburg, Germany (coordinates: 48.994129 N, 12.091213 E). Here, we took advantage of regularly spaced concrete slabs, which had been placed onto a grassy field to encourage *L. niger* colonies to settle underneath. The slabs had been in place for over 5 years, and all had been colonised by *L. niger*. These slabs serve as a source of experimental ants for behavioural experiments. Regular reinforcement of lab colonies, preceded by aggression tests, demonstrated that colony identity has been stable for many years. As such, we could well expect ants from neighbouring colonies to have extensive experience with each other (Czechowski, 1984). Eleven colonies, in a roughly linear array (see Figure 1), were selected for this study. We chose both end colonies (#1 and #11) and the central colony (#6) as target colonies (see Figure 1). Five ants from each target colony were tested individually against five ants from all other colonies, resulting in five pairs of ants per colony combination, with colonies 1 and 11 having one direct neighbour colony tested (2 and 10, respectively), and colony 6 having two (5 and 7). In addition, five pairs of ants from each target colony were tested (nestmates), and five heterospecific interactions per target colony were tested, using *Formica rufibarbis*, also found on the University of Regensburg campus.

Experimental procedure

To reduce observer bias, two experimenters conducted the aggression assays. One experimenter randomised the testing and collected ants from the colonies for each test session. The other remained completely blind to all ant identities.

Two test sessions were conducted per day, one in the morning and one in the afternoon. Each test session began by collecting ants from the respective test colonies (decided *a priori*) from the outside slabs and returning them to the lab. We chose to test aggression immediately, rather than allow acclimatisation to the lab, as one factor affecting intraspecific aggression in ants is differences in colony odour derived from their nesting substrate (Heinze et al., 1996). Keeping the ants in artificial nests in the lab may have eliminated a potentially important cue for the ants. As we conducted five encounters per colony pair, five ants of each colony per target–competitor pair were collected, along with some additional ants so that the tested ants would never be alone before testing. Ants were encouraged to leave the nest by briefly raising their nesting slab, causing ants at the surface to exit the nest onto the slab surface, from where they could be collected. Unused ants were released after each test session. Collected ants were stored in fluon-coated boxes with pieces of paper acting as a shelter. No less than 15 min and no more than 2 h elapsed

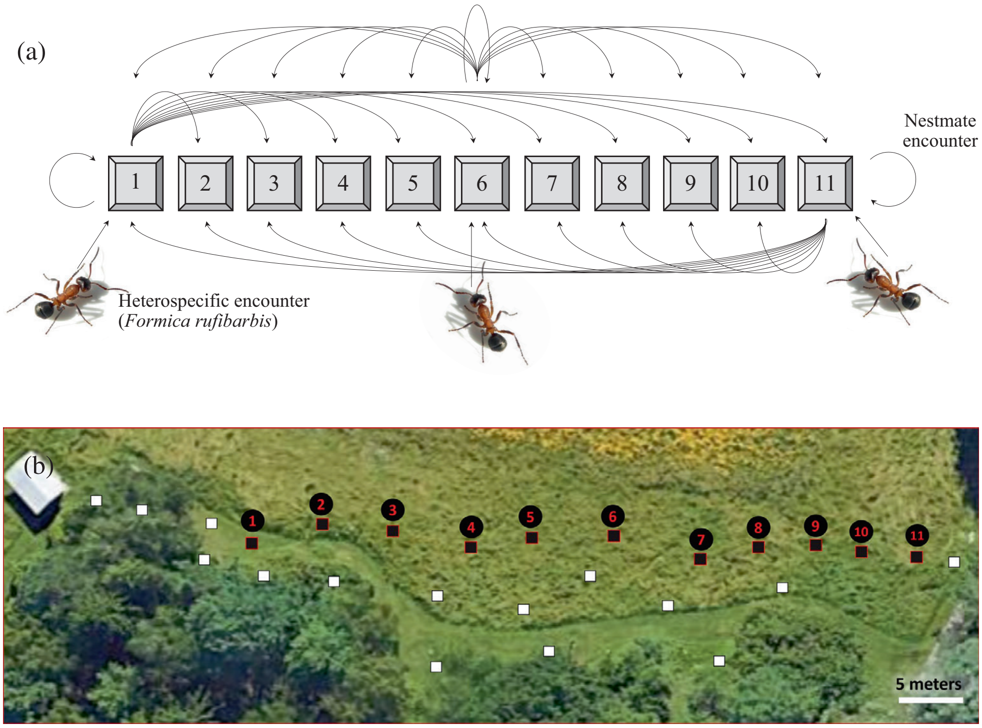

FIGURE 1 (a) Encounter schematic (36 combinations × 5 replicates = 180 pairwise encounters tested). Each square represents a colony in the linear array. Colonies 1, 6 and 11 are target colonies, and encounter each of the other colonies in the array, as well as ants from their own colony (nestmate encounters) and *Formica rufibarbis* (heterospecific encounter). Each arrow represents 5 pairwise encounters. (b) Position of all slabs in the test field. Non-numbered slabs were not used in this experiment.

between collection and testing. Distances between each slab and its neighbour were measured in centimetres from the centre of the slabs, using a field tape measure.

Ant pairs were placed separately in two halves of a divided arena, consisting of a plastic container (floor diameter 65 mm and wall height 25 mm), with the floor covered with a disposable graph paper inlay. The chamber was divided using a piece of fluon-coated acetate sheet. Ants were allowed a 30 s habituation period, after which the divider was removed. The ants were then free to interact for 2 min, and the behaviour of the ants was scored in real time. We scored all behaviours, but not the identity of the ant performing the behaviour. Both experimenters recorded the number of occurrences of each of the behaviours separately. However, these rarely diverged. On the rare occasions where counts diverged, these were averaged. A set of eight interactions were defined and noted *a priori* (see Table 2). However, fight duration and avoidance were not analysed and were excluded from this study, since prolonged fights were very rare (see below). However, when they did occur, they continued for the entire length of the recording session. Fighting duration was removed because extended fighting events were rare (only 10 fights longer than 1 s). Avoidance was removed because it was recorded only six times. After the first day of the experiment, we added a seventh interaction type *post hoc*, neutral body contact, which we considered to be an informative behaviour about the lack of aggression between two individuals. After 2 min of observation, each individual was placed separately in a 1.5 mL Eppendorf Safe-Lock Tube. Each tube also included a label

TABLE 2 Behaviours recorded.

Behaviour	Definition
Neutral body contact	Ants have body contact or are in the range where bodies could have contact but do not interact otherwise
Antennation	Antenna quickly move up and down while either also touching the other individual or the individual is approximately the length of an antenna away from the other
Jerking	Very fast back and forth movement of the whole body while staying in the same place
Mandible gaping	Mandibles open widely
Brief biting	Ant briefly (<1 s) bites the other, i.e., mandibles close around a body part of the other individual
Gaster flexing	Gaster is bend under the body pointing in front
Fighting/prolonged biting	Rolling up while biting each other and potentially spraying each other/at least one ant bites the other and holds on with her mandibles to a body part of the other for >1 s
Fighting duration (s)	Total time the ants were fighting or prolonged biting each other

that identified the date, the colony pair and, if possible, whether the respective ant was from the target or competitor colony. The tubes containing the ants were placed on ice for the remainder of the session (maximum 2 h) and after each stored at −20°C for later genetic analysis.

Note: All behaviours were defined *a priori*, except for neutral body contact, which was added on the second day of data collection. The number of times the behaviour occurred (except for Fighting duration, see Table 2 for details) was recorded independently by two experimenters. Note that avoidance behaviour and fight duration were recorded, but never analysed (see main text). Jerking and antennation can be seen in Video S1.

Molecular analyses

Due to resource limitations, only a subset of tested pairs underwent molecular analysis. We selected the pairs to be analysed by calculating the proportion of overtly aggressive behaviours (mandible gaping, brief biting, gaster flexing and fighting [in seconds) from the total behaviours recorded and then selecting the 22 pairs showing the least and most aggression. Note that this selection was performed *a priori*, well before formal statistical analysis. Since this selection did not cover all sampled colonies, several additional pairs were added to the molecular analysis where this was needed to cover the entire range of colonies with at least six individuals per colony. Finally, DNA was extracted from 5 to 22 workers in each of the 11 colonies (see Table S1 for details) using a CTAB method (modified from Green & Sambrook, 2012). Eleven highly variable microsatellite markers were used to determine the genetic relatedness and the colony structure: Ant1343, Ant3993, Ant 859, Ant 10878, Ant 575, Ant 8424, Ant 2794 (Butler et al., 2014), L10-282, L10-174, L1-5 and L10-53 (Fjerdingstad et al., 2003). Primer sequences are available in Table S2. For all markers, the 15 μL PCR reaction volume consisted of 7.5 μL GoTaq® G2 Hot Start Colourless Master Mix (M7433, Promega); 4.5 μL ddH2O, 1.0 μL unlabelled reverse primer (10 μM); 1.0 μL labelled forward primer (10 μM; labelled HEX, FAM and TET and the final concentration of 0.67 μM for each primer) and 1 μL DNA (2–10 ng). The PCR consisted of initial denaturation at 94°C (4 min), 33 cycles at 94°C (denaturation, 30 s), 55°C (annealing, 30 s) and 72°C (elongation, 30 s), and a final step at 72°C (1 min). The PCR products were analysed in an ABI PRISM 310 Genetic Analyser (PE Biosystems) after DNA denaturation at 90°C (1 min). Allele sizes were determined using the genescan 3.1 software (PE Biosystems). In case of PCR failure or unclear results, the molecular analysis was repeated to ensure that genotypic information was obtained for all individuals at all loci. All 11 loci were polymorphic and showed considerable variation with an average of 11.45 alleles across all samples from the 11 colonies (ant 1343: 6; ant 3993: 4; ant 859: 7; ant10878: 22; ant 575: 8; ant 8424: 5; ant 2794: 16; L10-282: 11; L10-174: 15; L1-5: 12 and L10-53: 20 alleles. Hardy–Weinberg Equilibrium was checked for at each locus using GenAlEx (Peakall & Smouse, 2006). For each locus, we recorded the sample size, the number of alleles, the effective number of alleles, the observed and expected heterozygosities, and the fixation index (GenAlEx). In addition, we confirmed the absence of stuttering and large allele dropout and estimated the frequency of null alleles (Microchecker 2.2.3; Van Oosterhout et al. 2004) (Table S4). Linkage disequilibrium was tested between each pair of markers (Genepop 4.7.5; Rousset 2008, Table S5).

The relatedness between pairs of colonies was calculated using the estimator of Queller and Goodnight (Queller & Goodnight, 1989) provided by GenAlEx. The Queller and Goodnight estimator (Queller & Goodnight, 1989) allowed us to determine the genotypic similarity of microsatellite markers between pairs of individuals compared with an expected value between two individuals taken at random from the population. Negative values indicated that the degree of kinship between the two individuals tested was less than that of individuals drawn randomly from the population. The relatedness between the two colonies was calculated based on the average relatedness of all pairs of individuals belonging to these colonies.

Statistical analyses

As a preliminary analysis, a first principal component analysis (PCA1) was conducted *a priori* on the seven behavioural variables before using the status of the interactants (nestmate, non-nestmate or heterospecific) as a classification variable. This analysis was used to ensure the consistency of the behaviours recorded during the interactions and to confirm that they varied properly according to the context. In effect, the allospecific interactions acted as the positive control (expected to show high aggression) while the nestmate interactions were the negative control (expected to show no aggression).

We then focused on the intraspecific, non-nestmate interactions ($n = 150$). Among the seven behavioural variables recorded, only the number of antennation and jerking events were not zero-inflated and were not *a priori* related to aggression. The number of each of the five other behaviours (neutral contacts = no aggression, mandible gaping, brief biting, gaster flexing and fighting = higher aggression level) was highly zero-inflated and, therefore, difficult to analyse separately as dependent variables of models. We therefore used the *ade4* package (Dray & Dufour, 2007) to perform a second PCA (PCA2) on these five variables, to derive the most informative summary variable describing the aggressiveness of the focal ant. Visual inspection of the scree plots showed an inflexion point that justified retaining two factors, but only the first dimension explained more than 30% of the variability and was biologically meaningful (see results), and was, therefore, retained as an "aggressiveness level" variable in the subsequent analyses. Note that the variable was reversed in subsequent analyses so that higher numbers represented higher levels of aggression.

The impact of spatial distance (in centimetres) and genetic relatedness between the nests on the behavioural responses were analysed using three (generalised) linear mixed models (M1 associated with the number of antennation events; M2 associated with the number of jerking events and M3 associated with the level of aggressiveness). As the level of aggressiveness fitted a Gamma distribution (package *fitdistrplus*, Delignette-Muller & Dutang, 2015), M3 was adjusted using the Gamma family after the transformation of the variable towards a positive variable by adding the minimal possible value of 1.6. In all three models, the identities of the focal nest were included as random effects. The spatial distance between the nests and the genetic relatedness between them were included as dependent variables.

We then ran the three models a second time (M'1–M'3) but with the neighbour status of the pair of colonies as a dependent variable (neighbour adjacent colonies; non-neighbours, i.e., strangers: all other pairs). Because of an extremely high genetic relatedness between two colonies compared with all other colony pairs (genetic relatedness around 0.2, i.e., circa half siblings, whereas all other pairs presented negative relatedness values varying between -0.3 and 0), we removed this one colony pair from all the models as it was heavily distorting the results. The results of the models including this pair are reported in Tables S6 and S7. All statistical analyses were carried out using the R v.3.5 software (R Core Team, 2018). All GLMM models were analysed using the package *glmmTMB* (Magnusson et al., 2020), and the quality of the model estimates was monitored using Pearson residuals using the DHARMa package (Hartig, 2020). Figures were created using the Effects package (Fox et al., 2016). For all statistical tests, the level of significance was set at $p < 0.05$.

RESULTS

The preliminary PCA1 (Figure 2) confirmed the relevance of the behavioural variables chosen. The allospecific interactions were characterised by more biting and fighting events, more mandible gaping and a few neutral body contacts compared with intraspecific interactions. Among the intraspecific dyads, the non-nestmates interactions showed more antennation, jerking and mandible gaping, whereas the interaction between nestmates showed more neutral body contacts.

Regarding the intraspecific interactions between the colonies (corresponding to the "non-nestmate" interactions of the previous analysis), the PCA2 (Figure S1) conducted on the five variables relating to aggressiveness (neutral contacts = no aggression, mandible gaping, brief biting, gaster flexing and fighting = higher aggression level) suggested that only the first axis should be maintained and fed into subsequent analyses. Indeed, the first dimension was relevant in terms of both contribution to the global variability (38.67%) and was biologically meaningful, as all the variables relating to aggressiveness were grouped on the left and opposed to the neutral contacts on the right, suggesting a strong division between the aggressive (left) and non-aggressive (right) interactions (Figure S1). The "aggression" variable was, therefore, derived from the first dimension of the PCA2.

The models confirmed that two of the three behavioural variables studied (M1: number of antennation events and M2: number of jerking events) were significantly impacted either by the spatial distance between the colonies or by the genetic relatedness between them, or both (Table 3). Specifically, as spatial distance increased, antennation decreased (M1: estimate = -1.030, $p = 0.009$), as well as the number of jerking events (M2: estimate = -1.394, $p = 0.034$; Figure 3). As genetic relatedness between the colonies increased, so did the number of jerking events (M2: estimate = 21.027, $p = 0.014$; Figure 3).

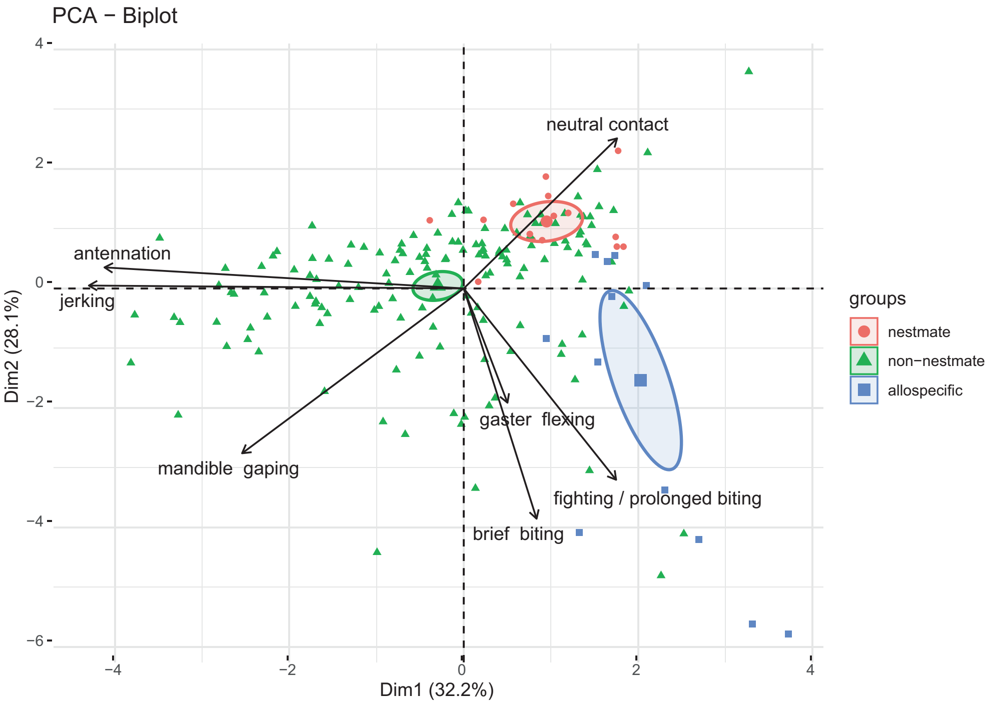

FIGURE 2 General PCA1 biplots incorporating allospecific (*L. niger* vs. *F. rufibarbis*) and conspecific (*L. niger* vs. *L. niger*) interactions. Axes referred to the score coordinate system with values related to the explained variance on each component. Ellipses are 0.95 confidence ellipses around group mean points.

TABLE 3 Effects of the distance (spatial distance between the colonies) and the relatedness (genetic relatedness between the colonies based on the Queller and Goodnight estimator) on the three behavioural descriptors: M1: number of antennation events, M2: number of jerking events and M3: aggression level.

M1: Antennation	Estimate	Std. error	*Z* value	*p* value
Intercept	11.930	0.908	13.144	<0.001
Distance	−1.030	0.397	−2.597	0.009
Relatedness	7.956	5.140	1.548	0.122
M2: Jerking	**Estimate**	**Std. error**	***Z* value**	***p* value**
Intercept	14.795	1.509	9.807	<0.001
Distance	−1.394	0.660	−2.115	0.034
Relatedness	21.027	8.544	2.461	0.014
M3: Aggression	**Estimate**	**Std. error**	***Z* value**	***p* value**
Intercept	0.302	0.144	2.092	0.036
Distance	−0.046	0.067	−0.690	0.491
Relatedness	−1.294	0.814	−1.590	0.112
M'1: Antennation	**Estimate**	**Std. error**	***Z* value**	***p* value**
Intercept	10.733	1.241	8.650	<0.001
Neighbour status	−0.077	1.313	−0.059	0.953
M'2: Jerking	**Estimate**	**Std. error**	***Z* value**	***p* value**
Intercept	11.400	2.071	5.505	<0.001
Distance	0.056	2.192	0.026	0.980
M'3: Aggression	**Estimate**	**Std. error**	***Z* value**	***p* value**
Intercept	0.396	0.197	2.013	0.044
Distance	0.132	0.208	0.633	0.523

Note: Effects of the neighbour status (binary variable) on the three behavioural descriptors: M'1: Number of antennation events, M'2: Number of jerking events and M'3: aggression level.

However, genetic relatedness did not impact the number of antennation events (M1: estimate = 7.956, *p* = 0.122). Aggression was not significantly predicted by either spatial distance or relatedness (M3: spatial distance; estimate = −0.046, *p* = 0.491; relatedness; estimate = −1.294, *p* = 0.112; Figure 3). It slightly trended to decrease as genetic relatedness increased. None of the three behavioural variables studied (number of antennation events, number of jerking events and level of aggressiveness) were impacted by the neighbour status of the colonies (M1'–M3', Table 3). Spatial distance and relatedness were not correlated (estimate = 0.00543, *p* = 0.410).

DISCUSSION

The behaviours we measured captured differences in aggression among nestmate, non-nestmate and allospecific encounters (Figure 2): encounters with allospecific ants were much more aggressive, according to the composite aggression score (Figure S1) than the encounters with either nestmates or conspecific non-nestmates. This implies that, perhaps surprisingly, some allospecifics represent a larger threat or competitor than conspecifics. Conversely (see below), *Lasius niger* may have developed intraspecific communication strategies to avoid costly fighting, although raids and fighting between the colonies of different sizes may be lethal (Seifert, 2007). Nestmate and non-nestmate encounters were mostly differentiated by increased jerking and antennation behaviours in non-nestmate encounters and increased neutral body contacts for nestmate encounters. This would seem to imply that jerking and antennation behaviour can be taken as a measure of moderate aggression, as assumed for this and other species previously (Devigne & Detrain, 2002; Holway et al., 1998). However, the pattern of these behaviours in interactions between the non-nestmates does not support this assumption: as spatial distance increases, antennation and jerking decrease. This requires a more nuanced interpretation of these behaviours.

Antennation can be assumed to be involved with information gathering. Nestmate recognition is driven by the ants' cuticular hydrocarbon profiles used for nestmate recognition, which are influenced both by genetic factors and extrinsic environmental factors (van Zweden & d'Ettorre, 2010). It is thus reasonable that antennation, and thus information gathering, increases for non-nestmates from spatially closer ants, as they will be harder to distinguish from nestmates—although we note that the opposite pattern could also have been explained, had it been found, by assuming more experience with closer ants. Jerking behaviour is more difficult to interpret. This behaviour is often observed being performed by active foragers returning to the nest (TJC, AK, unpubl. obs.). It can also often be triggered by allowing light to enter laboratory nests. Similar jerking behaviours have been reported in other ant species as recruitment signals (Hölldobler, 1971, 1976, 1983; Liefke et al., 2001) and as a response to light or puffs of air entering the nest (Weber, 1957). Between nestmates, it thus seems to represent a form of communication, potentially a generalised activity upregulation signal. This is supported by the fact that this behaviour is hardly ever directed towards allospecifics (6.6% of encounters) but is almost ubiquitous in conspecific encounters between non-nestmates (93.3% of encounters) and between nestmates (100%). In *Linepithema humile*, jerking behaviour has been reported to be more common between nestmates after feeding on higher quality food (Sola & Josens, 2016). It is less clear what this jerking behaviour means between non-nestmate conspecifics. It has been reported to play a role in tournaments between ants in *Lasius niger* (Czechowski, 1984). According to Devigne and Detrain (2002), jerking behaviour is more common between non-nestmates than nestmates, and they assume that jerking behaviour between non-nestmates is a form of low-level aggression. Given this and the pattern of decreasing jerking and antennation with decreasing relatedness and increasing spatial distance we describe, we tentatively propose that the combination of antennation and jerking can be approximated to 'negotiation,' where ants are gathering information about each other and attempting to avoid overt aggression. In a normal encounter, the ant pairs would be able to communicate, 'negotiate' and then withdraw. However, withdrawal was not possible in our

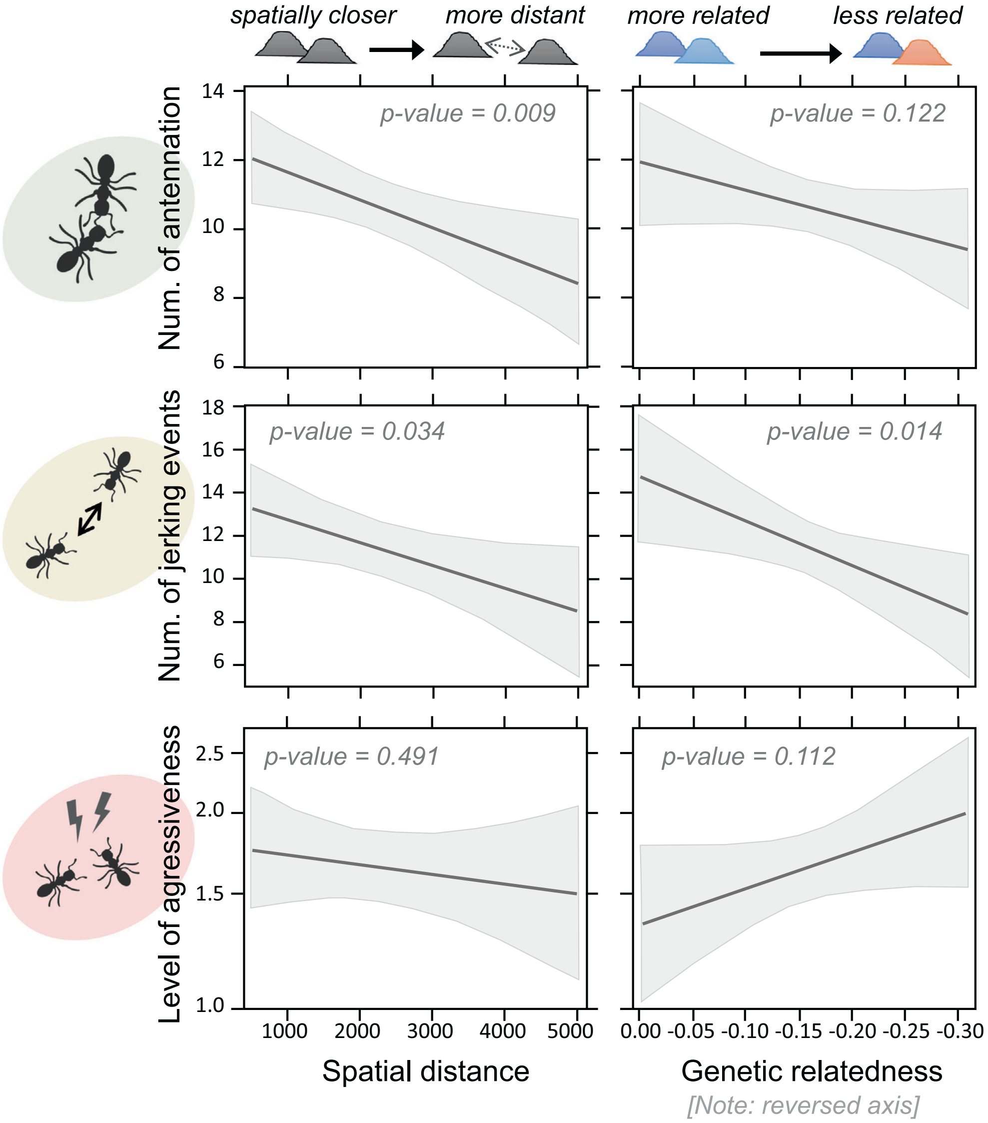

FIGURE 3 Results of the predicted effects for the three models M1–M3, investigating the relationship between the behaviour observed during the encounter and the spatial distance (left) and the genetic relatedness (right) between the colonies ($n = 150$). The figure provides predictions for the response variable (*Y*-axis) across the range of values of each explanatory variable (*X*-axis), whilst holding values of the other explanatory variable constant. The confidence band (shaded—95% CI) and the regression line (bold) have been calculated based on the values predicted by the models. Note that the genetic relatedness axis has been reversed to make spatial distance and genetic relatedness easier to compare.

setup, potentially resulting in the eventual occurrence of aggression. This would explain why aggression does not correlate with these 'negotiation' behaviours. Our results imply that jerking and antennation may be good measures of moderate aggression, before more overt and traditional signs of aggression (mandible gaping and gaster curling) appear. Antennation also no doubt plays an important role in distinguishing nestmate from non-nestmate. If both ants are nestmates, no negotiation is required.

Importantly, we found no evidence for neighbour–stranger response differences (NSRDs) in this system—neither in terms of aggression nor in terms of jerking or antennation: direct neighbours were not responded to in a different manner from non-direct neighbours. This absence of NSRDs could be because, in this system, neighbours and strangers present equivalent threat levels or a lack of differentiation in threat levels posed by neighbours and strangers. The latter case could be partly explained by the homogeneous environmental conditions within the studied area. In Cordonnier et al. (2022), higher levels of aggression were observed between allopatric individuals compared with individuals sharing similar environmental characteristics. Here, the relative similarity between nests in terms of substrate or available food could also have induced a homogenisation of cuticular hydrocarbons, with a consequent reduction in recognition of non-nestmates and aggressiveness (van Zweden & d'Ettorre, 2010). Nonetheless, such an environmental impact is not consistent in the literature, with some studies on the relationship between environment and aggressive behaviours suggesting correlations (e.g., Benedek &

Kobori, 2014; Frizzi et al., 2015) while others do not (e.g. Langen et al., 2000; Martin et al., 2012). However, absence of evidence is not evidence of absence. Aggression in ants often varies strongly with season (Ichinose, 1991; Katzerke et al., 2006; Thurin & Aron, 2008), as raiding of conspecifics for brood and territorial disputes over valuable food resources likely occur mostly in spring (Czechowski, 1984). In *Plagiolepis pygmaea*, significant seasonal variations are expected in the levels of aggression among workers of different colonies according to the biological cycle of the species (Thurin & Aron, 2008). In *Formica exsecta*, aggression levels significantly correlated with spatial distance between nests in spring, but not in summer or in autumn (Katzerke et al., 2006). Aggression among *Formica polyctena* colonies is highest in the spring when nests become active and taper off in the summer, and indeed in autumn, neighbouring *F. polyctena* colonies share foraging trails without aggression (Mabelis, 1978). In *Paratrechina flavipes*, workers were aggressive to related individuals only during the season when the nest was active (Ichinose, 1991). It is thus reasonable to expect that such a seasonal effect could also occur in other species, including *L. niger*, which hibernates from October until the end of March, inducing a Nasty Neighbour effect (higher aggression to direct neighbours) that might manifest only in spring. However, we think it unlikely that a Dear Neighbour effect (lower aggression to direct neighbour) would manifest at different seasons, and no record in the literature suggests such a pattern. The experiment was conducted in high summer, which should be the ideal time to detect a Dear Neighbour effect once territorial disputes are concluded. Thus, while we are not confident about excluding a Nasty Neighbour effect, we believe a Dear Neighbour effect is unlikely to play a role in *L. niger*. Finally, an important caveat is that we did not attempt to locate all *L. niger* colonies in the area, and we did not include all the known colonies in the area in the experiment (Figure 1b). Thus, it is possible that what we consider to be direct neighbours may have had a buffer colony in between them. Added to this is the fact that some colonies likely had more neighbours than others, potentially influencing NSRDs. For example, in *Iridomyrmex purpureus*, aggression towards non-nestmates was influenced by the density of surrounding conspecific nests, inducing more aggressive behaviour when nest density was higher (Thomas et al., 1999). NSRDs also occur between different species, such as between the dominant *Formica integroides* and the submissive *F. xerophila* (Tanner & Adler, 2009). Considering the local ant community and interspecific aggression could, therefore, provide more information, as *L. niger* shows a stronger dominance and aggressiveness towards other species, allowing a better differentiation of NSRDs at the interspecific level. All experiments are a trade-off between ecological realism and tight control: the current experiment is a field study which, although taking advantage of a semi-regular array to increase control, nonetheless cannot guarantee full control.

Finally, it must be mentioned that due to limited resources, the study had a reduced power. Likewise, we lack data on neutral body contact from the first day of data collection. We note a non-significant trend for antennation to drop with relatedness and aggression to rise. While we remain ambivalent about whether these represent biologically meaningful patterns, we again caution that an absence of evidence is not evidence of absence.

The fact that aggression did not correlate with spatial distance or relatedness, but that antennation and jerking did, highlights the importance of considering non-overtly aggressive behaviours when examining neighbour–stranger response differences, or the correlation of responses to physical and relatedness. While the theoretical framework for the field of NSRDs arises out of competition research (Temeles, 1994), it could also be used to examine the strategies used by animals to avoid aggression, as proposed here. Indeed, perhaps more emphasis needs to be given to increased cooperation between neighbours, which is expected both theoretically (Eliassen & Jørgensen, 2014; Getty, 1987) and observed empirically (Booksmythe et al., 2010; Elfström, 1997).

Overall, we find evidence that jerking and antennation behaviours are better measures for describing non-aggressive or pre-aggressive interactions among conspecifics than traditional measures of aggression such as biting and mandible gaping. These behaviours decrease with physical distance and increase with relatedness. We propose, as a working hypothesis, that these behaviours together can be considered 'negotiation' behaviour. Future studies, in which ants have the possibility of escaping, could shed light on this idea. We found no evidence of either a Dear neighbour or a Nasty Neighbour effect, although for the latter, we suggest future studies should evaluate whether neighbours and strangers present varying degrees of threat and explore the occurrence of a potential "Dear enemy" effect in Spring. While physical distance and relatedness affect behaviour during encounters in the ecologically important ant *L. niger*, NSRDs do not seem to play a major role in their behavioural ecology.

AUTHOR CONTRIBUTIONS

Tomer J. Czaczkes: conceptualisation, methodology, writing- first draft, funding aquisition, supervision. **Alexandra Koch:** Methodology; investigation; writing – review and editing; validation. **Sophie Schmid:** Investigation. **Andreas Trindl:** Methodology; validation. **J. Heinze:** Validation; writing – review and editing; supervision. **Marion Cordonnier:** Visualization; writing – review and editing; funding acquisition; formal analysis.

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CONFLICT OF INTEREST STATEMENT

Tomer J. Czaczkes is currently an Associate Editor for Ecological Entomology. The authors declare no other conflicts of interest.

DATA AVAILABILITY STATEMENT

The Genotype raw data generated during the current study, the complete dataset used for analyses, and the entire code and output for the analysis are available in the Zenodo repository [10.5281/zenodo.7767733].

ETHICS STATEMENT

None of the insects used in this study are protected by law, and no ethical oversight is required to work with these animals. All unused animals were returned to their colony of origin.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Overall description of the genotyping results for the 11 studied colonies. The genotypes of mated queens and males were inferred in each colony based on worker genotypes. At each locus, two alleles shared by all the workers were assigned to the queen, while the putative genotypes of the haploid fathers were determined from the alleles unassigned to the mother. This pattern was iterated over the 11 markers until reaching a minimal number of males. The inferred genotypes of the mates allowed the calculation of within-nest relatedness between them.

Table S2. Sequences, alleles number and size range of the 11 microsatellite primers used in the study.

Table S3. Chi-squared test of Hardy–Weinberg equilibrium for the 11 primers and the 11 colonies studied. M: Monomorphic.

Table S4. Detection of stuttering, large allele dropout, null allele and null allele frequencies (Microchecker 2.2.3), sample size, number of alleles and effective alleles, observed and expected heterozygosity, fixation index (GenAlEx) for each of the 11 microsatellite markers.

Table S5. Linkage disequilibrium between each pair of the 11 microsatellite markers (Genepop 4.7.5).

Table S6. Effects of the Distance (spatial distance between colonies) and the Relatedness (genetic relatedness between colonies based on the Queller and Goodnight estimator) on the three behavioural descriptors: M1: Number of antennation events, M2: Number of jerking events, M3: aggression level for the models incorporating the interactions between the two colonies genetically highly related.

Table S7. Effects of the neighbour status (binary variable) on the three behavioural descriptors: M'1: Number of antennation events, M'2: Number of jerking events, M'3: aggression level, for the models incorporating the interactions between the two colonies genetically highly related.

Figure S1. PCA1 biplot conducted on the 5 variables relating to aggressiveness (neutral contacts = no aggression, mandible gaping, brief biting, gaster flexing and fighting = higher aggression level). Axes referred to the scores coordinate system with values related to the explained variance on each component.

Video S1. Antennation and jerking.

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