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Seasonal nestmate recognition in the polydomous ant *Plagiolepis pygmaea*

NICOLAS THURIN & SERGE ARON
Behavioural and Evolutionary Ecology, Université Libre de Bruxelles

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Nestmate recognition cues can derive from genetic and/or environmental factors and can be context-dependent rather than fixed over time. We examined the influence of genetic relatedness and environment on nestmate recognition and its seasonal variations in a natural population of the polydomous (multiple-nests per colony) ant *Plagiolepis pygmaea* in southern France. Recognition between colonies was measured by testing aggression levels during encounters between five workers of colony A and one of colony B and vice versa. The combination of genetic data, spatial data and aggressive behaviour data shows that nestmate recognition cues have principally a genetic component. Whereas workers from different nests of the same colony are never aggressive to each other, they are always hostile to alien conspecifics regardless of the spatial or genetic distance between the colonies. Our results also reveal significant seasonal variations in the levels of aggression among workers of different colonies, probably according to the biological cycles of the species. Surprisingly, despite the mode of colony reproduction being budding in *P. pygmaea*, the population of Tarabel is not genetically structured.

Keywords: ants; nestmate recognition; *Plagiolepis pygmaea*; polydomy; seasonal variation

Nestmate recognition is a critical element in the evolution and maintenance of cooperative social groups (Fletcher & Michener 1987; Hepper 1991; Pfennig & Sherman 1995). In social insects, discrimination of nestmates from alien conspecifics and preservation of colony integrity rely on the use of chemical odour cues present on the cuticles of individuals (Field 1905; Forel 1923; Le Masne 1952). These recognition cues may have a genetic basis, they may be derived from the environment (e.g. diet or nesting material), or they may originate from both sources (Wilson 1971; Jutsum et al. 1979; Carlin & Hölldobler 1986; Gamboa et al. 1986; Obin & Vander Meer 1988; Crosland 1989; Hölldobler & Wilson 1990; Le Moli et al. 1992; Heinze et al. 1996; Liang & Silverman 2000). Aggression assays are useful to investigate nestmate recognition and its genetic and/or environmental origin in social insects (Gamboa et al. 1991; Roulston et al. 2003). When recognition cues are environmentally determined, individuals from nearby nests share a large proportion of chemicals and are expected to be less aggressive to each other than towards conspecifics from more distant nests. When recognition cues are genetically determined, individuals from related nests share more recognition cues and are expected to be less aggressive to each other than towards unrelated individuals, whatever the geographical distance between the colonies or their environmental conditions. Evidence for environmentally or genetically determined kin recognition cues in natural populations comes from several ants, bees and wasps in which aggressiveness was positively associated with the spatial distance between nests (Breed 1983; Gamboa et al. 1986; Gamboa 2004) or negatively associated with the relatedness between protagonists (Crozier & Dix 1979; Mintzer 1982; Waldman et al. 1988; Bennett 1989; Provost 1991; Beye et al. 1997, 1998; Panek & Gamboa 2000).

In social Hymenoptera, aggression as a measure of nestmate recognition is context dependent rather than fixed over time (Rosengren et al. 1985; Ichinose 1991; Suarez et al. 2002; Thomas et al. 2005; Katzerke et al. 2006). Seasonal variations in aggression towards foreign conspecifics in response to changing environmental

Correspondence: N. Thurin, Behavioural and Evolutionary Ecology, CP 160/12, Université Libre de Bruxelles, Avenue F.D. Roosevelt 50, B-1050 Brussels, Belgium (email: nthurin@ulb.ac.be).

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conditions have indeed been reported in some ants and bees. For example, under periods of food shortage increased competition results in higher levels of aggression towards nonnestmates to monopolize resources or prevent intercolonial food robbing (Downs & Ratnieks 2000). Temporal variations in aggressiveness may be determined by both extrinsic factors (e.g. temporal environmental stochasticity, variation in availability or quality of food across patches, temperature) and intrinsic factors (e.g. reproductive cycle, local worker density, within-nest patterns of relatedness).

In this study, we examined the influence of genetic relatedness and environment on nestmate recognition and its seasonal variations in a natural population of the ant *Plagiolepis pygmaea*. This species is highly polygynous (several queens per colony) and forms large polydomous colonies comprising several nests between which queens, workers and brood are exchanged (Passera 1963a). We combined genetic data and aggressive behaviour data for three seasons (spring, summer and autumn). First, we determined the level of aggression between individuals belonging to different nests of the same or a different colony and tested for seasonal variations in aggressiveness. Second, we analysed how aggression varies with the genetic distance and the spatial distance between nests. Finally, we examined the impact of environmental cues on nestmate recognition in two ways: (1) by analysing the aggression during encounters between workers from the same nest location but excavated in different years and (2) by studying the influence of alimentation on aggressiveness by feeding nestmate and nonnestmate workers with identical or different diets under laboratory conditions.

METHODS

Sampling

Plagiolepis pygmaea occurs throughout the southern part of Europe. It is commonly found in arid areas with low vegetation such as south-facing embankments with vertical slopes along roads. The number of nests per colony varies with the season, a process known as seasonal polydomy (Snyder & Herbers 1991). In spring, colonies split into numerous nests, between which workers, brood and queens are exchanged. In late summer, the nests recoalesce with other colony subunits, thus reducing the level of polydomy for overwintering. Colonies may contain up to several hundred queens and 150–5000 workers (Passera 1963b). Queens of *P. pygmaea* mate multiply (polyandry), with mates ranging 1–6 (Trontti et al. 2007).

Thirty *P. pygmaea* nests including queens, workers and brood were collected in October 2004, with 23 nests in July 2005 and 12 nests in April 2006, along a 168-m-long embankment located at Tarabel (Toulouse, southern France). Nests were collected all along the study area; the distance separating the nests excavated ranged from 0.6 to 168 m (Fig. 1). In the laboratory, individuals were immediately settled in experimental nests (Passera 1969) and fed honey and water ad libitum. Preliminary assays on a subsample of colonies not used here showed that other diets, including cockroaches, maggots, coleopteran larvae, eggs, meat, tuna or fruits, were refused and that only honey was eaten by ants. The experimental nests were maintained in the dark at $25 \pm 2^{\circ}$C from March to October, followed by an artificial hibernation at $11 \pm 1^{\circ}$C from November to February. They were kept under these laboratory conditions up to 20 months.

Behavioural Analysis

Aggressiveness between workers was tested in a neutral arena (fluon-coated petri dish of 2.5 cm diameter), following the procedure of Roulston et al. (2003). One worker from a nest A was placed inside a central, fluon-coated, open-bottomed tube of 0.6 cm diameter within the arena and five workers from a nest B were placed in the arena outside the tube. After 30 s, the central tube was removed and the behaviour of the ants was recorded for 3 min. Workers from the same nest served as controls. The behavioural interactions upon meeting were classified into five levels: (1) antennation, brief or repeated physical contact with the antennae tapping somewhere on the other ant; (2) intimidation, mandible opening and/or dorsal flexion of the gaster as escalation to chemical defence; (3) biting; (4) gaster application, ventral flexion of the gaster as deposition of formic acid; (5) fight, body tangling involving biting, gaster application and prolonged aggression. Level 1 is referred to as nonaggressive behaviour and levels 2–5 are referred to as aggressive behaviour. The arena was carefully cleaned with 70% ethanol after each encounter to remove potential contamination with formic acid or other chemicals.

For each pair of nests tested, we performed 10 replicates: five replicates in which one worker from nest A encountered five workers from nest B and five replicates in which one worker from nest B encountered five workers from nest A. Different workers were used for each trial. The highest level of aggression was recorded in each replicate. To test whether aggressiveness differed between the two sets of five replicates (five A–one B versus five B–one A), we first calculated the absolute value of the difference between the average maximal level of aggression in each of the two sets. This procedure was repeated for each pairs of nests. Then, the mean of the absolute values over all combination of nests was compared with that obtained after 10 000 randomizations of the data. Experimental and theoretical values were not significantly different (Student's t test: $t = 0.995$, $P = 0.32$), so that the scores of each set of 10 replicates were pooled.

To examine seasonal variations in aggressive behaviour, we analysed confrontations between workers from the same or different nests collected at different periods of the year: 61 pairs of nests were tested in autumn (October 2004), 38 in summer (July 2005) and 24 in spring (April 2006), representing a total of 1230 tests of aggression. Confrontations were performed within 3 weeks following collection. For each season, the level of aggression was plotted against the spatial or the genetic distance among nests.

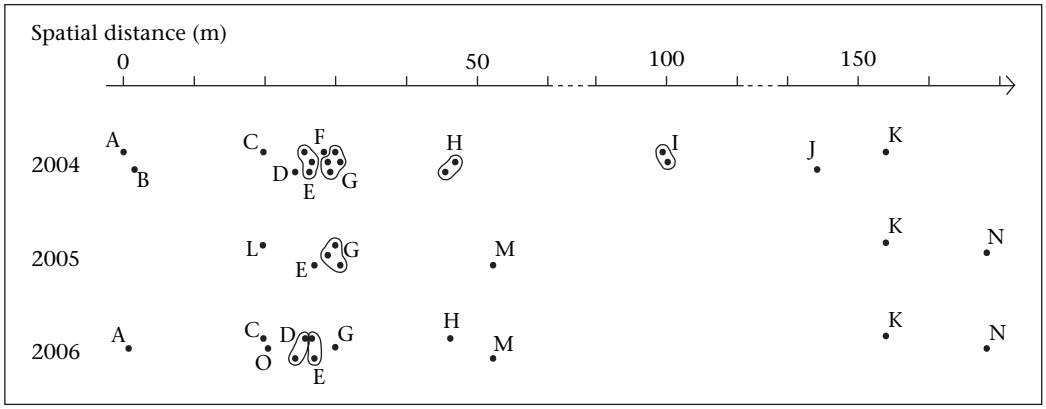

Figure 1. Distribution of *P. pygmaea* nests sampled in the population of Tarabel in 2004 (autumn), 2005 (summer) and 2006 (spring). Each point represents a single nest. Nests belonging to the same colony are surrounded; a letter is assigned to each colony.

To test the effect of environmental conditions on aggressive behaviour, we performed encounters between workers from the same colony location but excavated in different years. One colony was sampled in each year (2004, 2005, 2006), three colonies were sampled in 2 years (2004, 2006) and five colonies were sampled in 2 successive years (two colonies in 2004, 2005 and three colonies in 2005, 2006). Therefore, encounters opposed freshly collected workers to nestmates kept under laboratory conditions for 9–18 months. Genetic analyses (see below) confirmed that workers collected in the same location during the 3 years belonged to the same colony.

To test the influence of diet on nestmate recognition, nine nests collected in April 2006 were split into subunits of 200 workers and one to three queens. Twenty-eight of these experimental subunits were fed pure honey (Delhaize, Brussels, Belgium; 3.1% protein, 96.8% carbohydrate, 0.1% lipid), eight were given protein-enriched honey (addition of 0.2 mg proteins Optimum Nutrition (Aurora, U.S.A.) per mg; 10.9% protein, 88% carbohydrate, 1% lipid) and seven received lipid-enriched honey (addition of 0.11 mg oil Lesieur (Asnières sur Seine, France) per mg; 2.7% protein, 85.3% carbohydrate, 12% lipid). Workers from the same or different subunits were confronted 0–2 weeks, 3–5 weeks and 6–8 weeks after the dietary treatment was initiated. Twenty-two pairs of experimental units were tested three times each.

Overall, we examined 3220 confrontations between pairs of nests. Behavioural assays were performed blind as we determined the genetic composition of nests after behavioural analyses.

DNA Isolation and Microsatellite Analysis

Genotypes of 11–24 workers from each of 38 nests ($N = 18$ in 2004, $N = 8$ in 2005 and $N = 12$ in 2006) were scored at six microsatellite loci (P01, P06, P07, P20, P23 and P25; Trontti et al. 2003). The DNA was extracted from finely ground samples by incubating 90–120 min in 40 μL of Chelex (Bio-Rad) at 85°C. Samples were centrifuged for 30 s at 10 000*g*, and 2 μL of the supernatant was amplified by polymerase chain reaction following the fluorescent analysis protocols described in Trontti et al. (2003), using a PTC-200 thermal-cycler and Taq Gold polymerase (Fermentas). The amplified products were separated in an ABI capillary sequencer and sized against ROX-350 size standard (Applied Biosystems). The number of alleles scored at the six microsatellite loci ranged 3–8, with a mean expected heterozygosity $H_E = 0.50$ (0.42–0.54) and allele frequencies ranging 0–0.7.

Statistical Analysis

For all analyses, the contribution of colonies was considered equal. For statistical comparisons, the appropriate parametric or nonparametric test was used. Genetic distances (Weir & Cockerman 1984), level of heterozygosity and level of inbreeding were determined with SPAgeDi (Hardy & Vekemans 2002). Intranest relatedness was estimated using the program Relatedness (Queller & Goodnight 1989). For matrix correlation between spatial and genetic distances, we used Mantel test (Manly 1985) implemented in GeneAlEx 6 (Peakall & Smouse 2006) because the pairwise distance values between nests are not independent of each other. Because aggression was not tested for all nests the matrix of aggression was incomplete. Therefore, multiple regression analyses were conducted to test the impact of spatial and genetic distances on aggression. The spatial distance was logarithmically transformed (ln transformation; Zar 1996), and the genetic distance was estimated as $F_{ST}/1 - F_{ST}$ (Slatkin 1993). All *t* tests were two tailed.

RESULTS

Worker behaviour during encounters between individuals from different nests was homogeneous among replicates and was either friendly (mean aggression ± SD = 1.02 ± 0.05; range of the aggression level per pairs of nests: 1–1.2) or hostile (2.53 ± 0.57; range: 1.7–4.2) (Mann–Whitney *U* test: $U = 0$, $N_1 = 19$, $N_2 = 52$, $P < 0.0001$; Fig. 2a). Consistent with these observations, the genetic distance between nonaggressive nests was significantly lower (mean ± SD = 0.004 ± 0.016; range: −0.01 to 0.04)

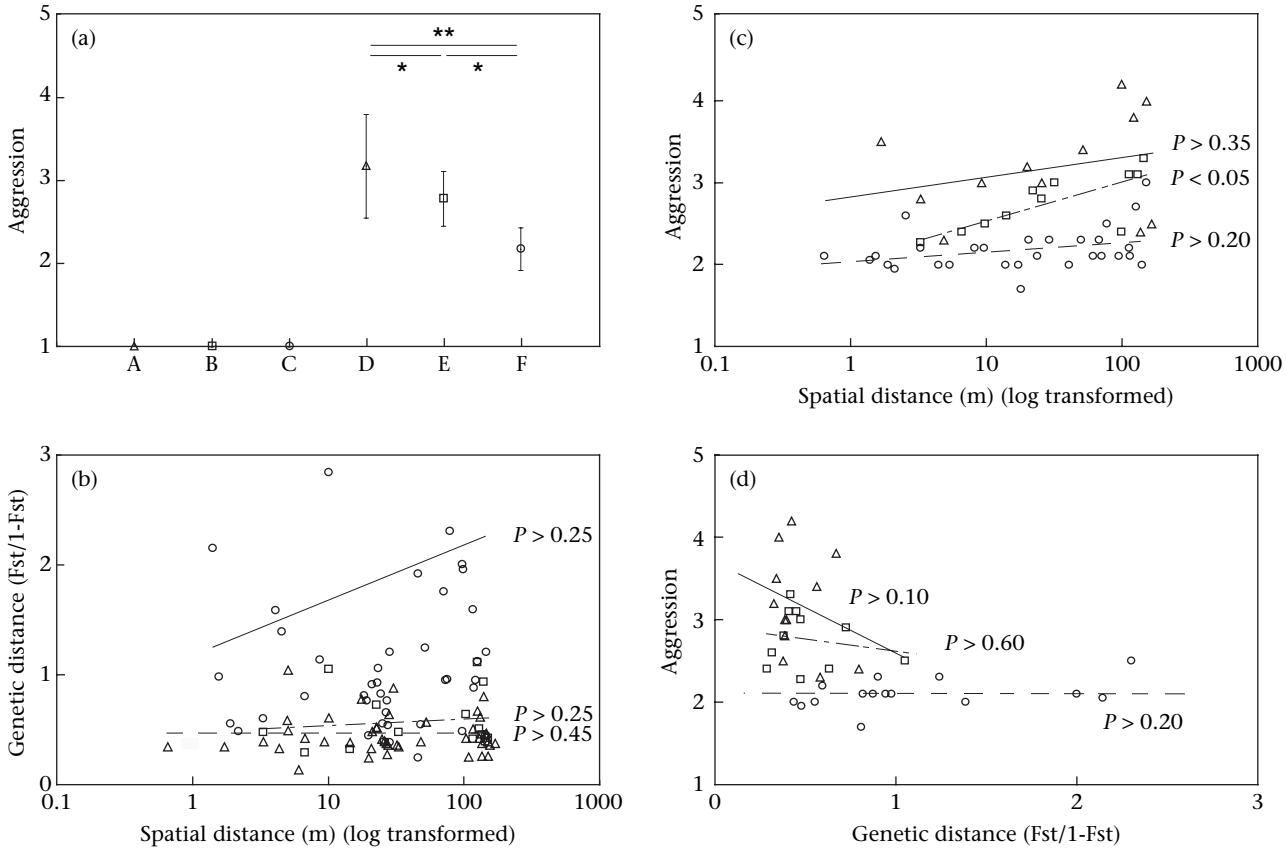

Figure 2. Variation in aggression with genetic distance, spatial distance and season (spring, summer and autumn) in the ant *P. pygmaea*. (a) Mean aggression between workers from different nests of a same colony (A, B, C) and between workers from different colonies (D, E, F), in spring (A, D), summer (B, E) and autumn (C, F) (ANOVA with Tukey post hoc test: $*P < 0.05$, $**P < 0.001$). (b) Relationship between spatial distance and genetic distance among colonies (Mantel test). (c) Level of aggression according to spatial distance between colonies. (d) Level of aggression according to genetic distance between colonies. Each point represents the mean score for one pair of colonies: △ spring, □ summer, ○ autumn. Regression lines are drawn for information only: - - - - spring; — — summer; — autumn.

than that between aggressive nests (0.79 ± 0.64; range: 0.29–3.31) (Mann–Whitney U test: $U = 0$, $N_1 = 19$, $N_2 = 39$, $P < 0.0001$). The combination of these behavioural and genetic data sets allowed us to assign the 38 nests to 15 distinct colonies.

Colony and Population Genetic Structure

The genotypes of 680 individuals (mean $\pm$ SD = 17.82 ± 3.75 workers per colony, $N = 38$ nests) were determined at six microsatellite loci. As expected from previous studies on other *P. pygmaea* populations (Trontti et al. 2005), the inbreeding coefficient in our study population was positive and significantly different from 0 ($F_{IT} = 0.29$, 0.39, 0.25 in autumn 2004, summer 2005 and spring 2006, respectively; Student's t test: $t > 38.5$, $P < 0.001$ for all seasons). This is consistent with limited dispersal of both sexes and the resulting intranidal mating between sexuals from the same colony (Passera 1969). The within-colony relatedness r ranged 0.22–0.77 and was on average equal to 0.51 (jackknife SE = 0.13). The within-nest relatedness did not differ from the within-colony relatedness (Mann–Whitney U test: $U = 112.5$, $N_1 = N_2 = 19$, $P > 0.25$), indicating that workers from different nests mix freely within a colony. The population was not genetically structured at the level of colonies. No correlation between the spatial and the genetic distance among colonies occurred, whatever the period of the year (Mantel test: $P > 0.25$ for all seasons; Fig. 2b). Thus, despite colony budding, neighbouring colonies were not more closely genetically related than distant colonies.

Effects of Season, Spatial Distance and Genetic Distance on Aggression

There was a significant seasonal effect on the aggression between colonies (ANOVA: $F_{1.45,0.07} = 20.46$, $P < 0.05$; Fig. 2a). Multiple comparisons showed that the level of aggression followed the sequence spring > summer > autumn (Tukey post hoc test: $P < 0.05$ for all comparisons). Therefore, our data were treated separately for each season in subsequent analyses.

Multiple regression analyses of aggression with the genetic and spatial distances indicated a significant effect in summer ($R^2 = 0.58$, regression $df = 2$, residual $df = 8$,

$P = 0.032$), with an impact of spatial distance ($P = 0.013$; Fig. 2c) but not genetic distance ($P = 0.643$; Fig. 2d) on aggressiveness. By contrast, no significant effects of genetic or spatial distance were found in spring ($R^2 = 0.21$, regression $df = 2$, residual $df = 9$, $P = 0.345$) and autumn ($R^2 = 0.24$, regression $df = 2$, residual $df = 12$, $P = 0.192$).

Effects of Environmental Conditions on Aggression

Encounters between freshly collected workers and their nestmates kept under laboratory conditions for 9 and 18 months were never aggressive ($N = 9$ and $N = 5$ pairs of nests, respectively; maximum aggression in all confrontations = 1). In the same way, workers originating from the same colony but kept separated in different laboratory nests for 9–18 months displayed no aggression to each other ($N = 12$ and $N = 9$ pairs of nests, respectively; maximum aggression = 1). By contrast, workers from different colonies reared under laboratory conditions for 9–18 months remained aggressive towards each other ($N = 23$ and $N = 6$ pairs of nests, respectively; range of the aggression level per pairs of nests: 2–4). Thus, the pattern of aggressiveness remains unchanged whatever the environmental conditions, rigorously identical or profoundly different, experienced by workers for several months.

The weak effect of alimentation on nestmate recognition was confirmed by feeding experiments involving different diets (honey, honey + protein, honey + lipid). There were no differences in the aggressiveness displayed during encounters between workers from the same experimental units fed differently ($N = 22$ pairs of nests) and those fed the same diet ($N = 18$ pairs of nests) for 0–2 weeks, 3–5 weeks or 6–8 weeks (maximum aggression in all confrontations = 1). Finally, workers from different colonies fed differently were not more aggressive towards each other than workers from different colonies fed with same diet for 6 weeks ($N = 15$ pairs of nests; Friedman test: $P > 0.80$).

DISCUSSION

The combination of genetic analyses and behavioural observations show that workers of the ant *P. pygmaea* discriminate nestmates from nonnestmates and that recognition cues have principally a genetic component. Workers from different nests of the same colony are never aggressive to each other, whereas they are always hostile to alien conspecifics regardless of the spatial distance between the nests. This antagonistic behaviour is also independent of the colony genetic distance. Indeed, worker aggressiveness is not a function of the relatedness between the protagonists; rather, it depends on the nestmate or nonnestmate status of the opponent. Furthermore, consistent with the genetic basis of nestmate recognition cues, confrontations between freshly collected workers and their nestmates collected 9 and 18 months earlier and reared under laboratory conditions are never aggressive. Thus, although workers experienced strongly different environmental conditions (natural versus artificial) and most probably belonged to different generations, this does not affect the outcome of encounters. Altogether, these results provide evidence that nestmate recognition cues and colony integrity are determined primarily genetically in natural populations of *P. pygmaea*.

Consistent with the genetic origin of recognition cues, results of our laboratory rearing study reveal that individuals from nests belonging to the same colony remain peaceful towards each other even when they are fed differently for several weeks, whereas individuals from nests from different colonies remain aggressive even when fed similarly for 18 months. However, whether the experimental diets used here affected the cuticular hydrocarbons of *P. pygmaea* workers remains unknown. Some diets have been shown to influence nestmate recognition in some ant species (e.g. Jutsum et al. 1979; Obin & Vander Meer 1988; Le Moli et al. 1992; Liang & Silverman 2000; Richard et al. 2004) but not in others (Jaffé & Marcuse 1983). Even geographical variations in the effect of the diet on cue expression and/or perception by other individuals were reported in the Argentine ant *Linepithema humile* (Buczkowski & Siverman 2006). Further chemical analyses should help to determine whether and how enriched honey affects cuticular hydrocarbon profiles in *P. pygmaea*.

Our results also reveal significant seasonal variations in the level of aggression towards foreign conspecifics. Aggressiveness is higher in spring, followed by summer and autumn. The seasonal variance in aggression of *P. pygmaea* workers might reflect a context-dependent adaptive behaviour in response to changing environmental conditions over the seasons. In this species, both male and female sexuals develop in summer from overwintering brood. Sexual larvae resume their growth in early spring, when food supplies are still limited; moreover, they require large amounts of proteins to achieve their development into adults (Passera 1969). In autumn, brood production decreases and worker activity is reduced (Passera 1969). Therefore, competition in spring when the food requirement is higher than the supply and, to a lesser extent, in summer is very likely stronger among the colonies than later in the year. To the best of our knowledge, such a change in the intraspecific acceptance threshold (Reeve 1989) linked with the nature of the brood reared and resource availability is documented in only a few other ant species (*Formica polyctena*, Mabelis 1979; *Formica truncorum*, Rosengren et al. 1985; *Paratrechina flavipes*, Ichinose 1991; *Linepithema humile*, Suarez et al. 2002; Thomas et al. 2005). In *F. polyctena*, Mabelis (1979) showed that intraspecific aggression is strongest in spring, at the onset of the reproductive period.

Although workers are always aggressive towards foreign conspecifics, the level of aggression increases with spatial distance of colonies in summer. This does not result from genetic viscosity because the study population is not spatially structured at any season. Two hypotheses are usually proposed to account for a positive association between aggressiveness and spatial distance. First, the 'dear-enemy phenomenon' predicts that territorial animals should display lower levels of aggression towards familiar neighbours than towards unfamiliar, more distant individuals (Temeles 1989; Bee & Gerhardt 2001). This behaviour

results in an abrupt transition in the level of aggressiveness between neighbouring and more distant colonies. In ants, the dear-enemy phenomenon has been acknowledged in some species (Gordon 1989; Langen et al. 2000; Knaden & Wehner 2003). However, it does not appear to apply to our study population because our data indicate a gradual increase in aggressiveness with spatial distance between colonies. Second, a correlation between aggression and distance may result from the influence of environmental factors on the recognition cues. Because workers from neighbouring colonies probably experience the same microhabitat, their odour cues are expected to be more similar. Consistent with this hypothesis, a strong effect of food quality and/or nesting material on nestmate recognition has been documented in several ants (Jutsum et al. 1979; Stuart 1987; Le Moli & Mori 1990; Le Moli et al. 1992; Heinze et al. 1996; Richard et al. 2004). Though our experiments do not indicate an effect of the environmental conditions on aggressiveness, one may not completely exclude that they affect nestmate recognition at some periods of the year. In this species, the positive association between aggression and distance occurs in summer. A possible explanation is that local environmental conditions are more heterogeneous in the study population and ants have access to a wide variety of food items that might influence colony odour in conjunction with genetic cues. By contrast, environmental diversity could be reduced in spring and autumn and odour cues derived from the environment could play only a minor role, if any, on nestmate recognition. In addition, as mentioned above, competition for access to resources is probably more intense in spring, when sexuals are reared to adulthood. At this time, genetically based recognition cues would be the prime mechanism to maintain the segregation of colonies. Whether this scenario holds under natural conditions awaits further study.

Our genetic analyses indicate a lack of genetic structuring in the study population. No isolation by distance could be detected. This is surprising, given that colonies of *P. pygmaea* reproduce by budding (i.e. young mated queens leave the colony with adult workers to initiate new colonies nearby), a process that should generate a strong population structure. Two nonmutually exclusive hypotheses may account for this result. First, it may stem from some gene flow between colonies, for instance if males and/or female sexuals integrate foreign colonies. However, gene flow is probably very low in *P. pygmaea* and should not significantly affect population structure. Previous genetic analyses indeed showed that mating proceeds in the nest between related individuals; not a single father was found to belong to a colony different from that of their queen's mate (Trontti et al. 2007). Moreover, independent foundation by freshly mated queens is extremely rare and unsuccessful in this species (Passera 1969). Second, the lack of genetic viscosity may result from the absence of genetic differentiation between the new buds and the maternal nest. In *P. pygmaea*, colony budding involves departure of several closely related queens and workers to find a new polygynous colony. As a consequence, allele frequencies do not differ between mother and daughter colonies. Because new buds are genetically highly representative of mother colonies, the founding effect commonly associated with budding may become blurred. The lack of population structure found in this study contrasts with previous studies on other populations of the same species (Trontti et al. 2005). This discrepancy probably results from differences in the sampling methods used in both studies. In their work, Trontti et al. (2005) regarded nests separated by more than 1 m as belonging to different colonies and population structure was determined by taking into account the genetic distance between nests. Our genetic and behavioural analyses show, however, that nests of a single colony may be separated by up to 1.7 m. As a consequence, genetically similar nests were sometimes considered erroneously as units belonging to distinct colonies by Trontti et al. (2005). It should be noted that a genetic structuring of the population also occurs in Tarabel when correlations are made at the scale of the nests rather than the colonies (Mantel test: $P = 0.22$, $P = 0.01$ and $P = 0.001$ in spring, summer and autumn, respectively).

In conclusion, this study shows that nestmate recognition is determined primarily by genetic factors in the ant *P. pygmaea*. However, an additional effect of environmental conditions on recognition cues might also occur at some periods of the year. It also shows significant seasonal variations in the level of aggressiveness that are closely linked with the biological cycle of the species. Finally, although colonies of the species reproduce by budding, the population studied is not genetically structured due to the important within-colony genetic diversity relative to the low population genetic diversity. More generally, this work stresses the importance of combining behavioural and genetic analyses at different periods of the year to determine the origin of nestmate recognition, how it varies over time and the population structure in polydomous species.

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