Development of Experimentally Orphaned Termite Worker Colonies of Two *Reticulitermes* Species (Isoptera: Rhinotermitidae)

by

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ABSTRACT

Survival and caste differentiation were observed under controlled conditions in orphaned experimental colonies of the subterranean termites *Reticulitermes grassei* and *R. santonensis*. Worker colonies had different sizes (30, 50, 100, 200 and 300); after 12 and 32 months the differentiation of colony members in other castes was observed. Twelve months after orphaning, 80% of the colonies had survived. For the two species, a mean number of one soldier was observed in 7 colonies and between 1 and 3 nymphs were present in 18 colonies, whatever the initial number of workers. In 53% of the surviving colonies, the differentiation of secondary reproductives occurred and they produced viable offspring. The external morphology of *R. grassei* male reproductives did not differ significantly from those of workers or nymphs. Thirty-two months after orphaning, colonies with an initial number of 30 workers were comprised of secondary reproductives and their offspring. In termite species, caste differentiation pathways and thus the caste system are highly flexible. Therefore, our results show that a small number of subterranean termites could establish a new colony within few years and thus invade a new habitat, for example in urban areas.

Keywords: subterranean termites, small colonies, survival, neotenics differentiation, urban habitat.

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INTRODUCTION

In eusocial insects, the colony is a social unit constituted of individuals, which are organised in different castes according to their behaviour, and/or their morphology. Colonies can be classified on the basis of the mode of foundation, the number of reproductives and the number of nest units forming a colony (Pamilo *et al.* 1997). The social organization of colonies (i.e. number of individuals, relatedness and reproductive skew among them) can vary within species or even within populations, and ecological and social factors can induce variations in the social organization of colonies (Ross & Keller 1995). In some Hymenopteran species, an example of such variation within a population is a difference in queen number per colony. Adoption of young queens can be favored when habitat saturation is high (e.g. *Myrmica sulcinodis* (Nylander): Pedersen & Boomsma 1999) or when ecosystems are characterized by interspecific competition and territoriality (e.g. *Ectatomma tuberculatum* (Olivier): Hora *et al.* 2005).

Unlike others social insects like bees, wasps or ants, Isopteran species have a highly plastic social organization. Termites are hemimetabolous insects and, except for the primary reproductives, all individuals are immature. They are organised in castes (workers, soldiers and nymphs developing into reproductives) which are an example of larval polyphenism (Nijhout 2003; Korb & Katrantzis 2004). The developmental pathways are not always irreversible (Noirot 1989). In some species of Termopsidae and Kalotermitidae, nest and food resources are situated in the same piece of wood (Abe 1987) and workers have a flexible development; they can switch to another caste or even develop regressively into a former instar, which is a unique developmental pattern (Korb & Katrantzis 2004). In numerous termite species, different social organizations occur within populations, as a result of the mode of foundation but also as a result of the flexibility of individual developmental patterns. Colonies can thus exhibit various breeding systems: foundation by one couple of primary reproductives (a queen and a king) or by several queens (polygyny) or kings (polyandry) (Fisher *et al.* 2004). If one of them dies (or both), immature individuals can become secondary reproductives: they have a larval morphology but their sexual organs are functional (Noirot 1956; Büchli 1958). These individuals are named ergatoid or nymphoid

neotenics when they develop from workers or nymphs respectively. Within a colony, they can be distinguished from workers or nymphs by a longer abdomen, darker pigmentation, slight sclerotisation and the presence of eyes and ocelli (Weesner 1965; Plateaux & Clément 1984; Krishna 1989; Serment & Tourteaux 1991; Thorne 1996, 1998). When neotenics replace the missing parent they are named replacement reproductives. Sometimes neotenics can also develop in the presence of primary reproductives and are named supplementary reproductives (Thorne 1996; Roisin 2000). In several termite species, the social organization can vary within a population but also within a colony during its lifespan, if the death of one or several primary reproductives occurs.

Some studies have focused on the origin of replacement reproductives and the ability of colonies to survive an orphaning. Lenz and Runko (1993) orphaned several field colonies of the Australian termite *Coptotermes lacteus* (Froggatt): primary queens were quickly replaced by nymphoid neotenics. Colonies produced a high number of nymphs all-year round. As the original colonies' survival was low, it was probably due to a strategy to disperse more alates. Pawson and Gold (1996) have investigated the caste differentiation in orphaned colonies of the American subterranean species *Reticulitermes flavipes* (Kollar), *R. virginicus* (Banks) and *R. hageni* (Banks) at 5 worker densities. Half of the colonies survived and their growth was ensured by the high reproduction potential of replacement reproductives. The social structure could vary between species: *R. virginicus* colonies produced more reproductives than did colonies of *R. flavipes* and *R. hageni*.

In Europe, a large majority of termite species are subterranean and belong to the genus *Reticulitermes*. Among them, *R. grassei* Clément and *R. santonensis* (Feytaud) have populations in natural environments in the south-western area of France and they cause severe damage to buildings in urban areas. The social structure of *R. grassei* and *R. santonensis* colonies within French populations has been analysed in natural and urban local conditions (DeHeer *et al.* 2005; Dronnet *et al.* 2005). The presence of colonies in urban areas is certainly due to human activity, particularly the transport of soil and infested wood. It is generally assumed that fractions of colonies are at the origin of the populations in built-up areas and their development can be achieved because

of the flexibility of the social structure within populations, particularly the differentiation of workers and nymphs into secondary reproductives.

The purpose of the present study was to evaluate the ontogenic potentialities of small orphaned groups of workers of the species *R. grassei* and *R. santonensis* isolated from their respective colonies. As in Pawson and Gold (1996), the development of new reproductives and their brood was observed in groups with different sizes of workers. Our first results were obtained over a period of 12 to 32 months. When neotenics and their offspring were observed, we collected them and tried to assess the number of parental pairs producing the new offspring with microsatellite markers.

MATERIAL AND METHODS

Collection of termites and rearing conditions

A colony of *R. santonensis* was collected in April 2000 on the island of Oléron (Charénte Maritime, France) and maintained in the laboratory until the experiment started. The colony of *R. grassei* was collected in July 2003 at Grenade-sur-l'Adour (Landes, France). In both cases the termites were initially kept in the original pieces of wood they had been feeding on in the field. Species identities were confirmed using cuticular hydrocarbon profiles (Bagnères *et al.* 1991; Clément *et al.* 2001, data not shown) and DNA sequences of the mitochondrial cytochrome oxydase II gene (Kutnik *et al.* 2004, data not shown). For the experiments, approximately 4100 workers of 5^{th} to 7^{th} instars were collected from each colony.

Orphaning experiment

For each species, five colony sizes were tested: 30, 50, 100, 200 and 300 workers, respectively named type 30, 50, 100, 200 and 300. Six artificial colonies (replicates) for each type were prepared, thus 30 artificial colonies per species. For each artificial colony, the termites were transferred in a plastic box (120 x 90 x 50 mm) containing 150 g of moistened Fontainebleau sand and a piece of poplar wood (20 x 20 x 20 mm). A new piece of wood was placed in the box before half of the food resource was consumed. The relative humidity was maintained at 80% inside each plastic box. During the experiment, artificial colonies were maintained under constant darkness, at room temperature.

After 12 months, we evaluated the number of surviving artificial colonies and the proportion of surviving workers (initially placed in the box and named 'old' workers) within these colonies. We recorded the numbers of new nymphs, soldiers and secondary reproductives as well as their eggs, young larvae and new workers when present in artificial colonies. All termites from each artificial colony were transferred in a new plastic box (120 x 90 x 50 mm) with Fontainebleau sand and a piece of poplar wood, and maintained in the conditions described previously.

After 24 months, almost all *R. grassei* artificial colonies of type 200 and 300 were surviving and several secondary reproductives with a relatively high number of eggs and young larvae were observed (cf. results). Thus we chose these artificial colonies to evaluate if females were sexually mature and to estimate the number of reproductives which produced the offspring. The other surviving artificial colonies (type 30 to 100 *R. grassei* replicates and *R. santonensis* artificial colonies) were kept in the same conditions to evaluate the development of small groups of termites 32 months after orphaning. Therefore, the experiment was divided in two parts:

I) The surviving *R. grassei* artificial colonies of type 30, 50, 100 and all the surviving artificial colonies of *R. santonensis* were maintained in the conditions of the experiment. After eight months (32 months after beginning), all the termites from surviving artificial colonies were collected, their caste determined, and offspring number evaluated.

II) The development of female neotenics was observed in *R. grassei* surviving artificial colonies of type 200 and 300. The female neotenics were isolated and their abdomens were dissected under a dissecting microscope. Their ovarian development was observed and the spermathecae were collected and flattened in a drop of Beadle buffer (128.3 mM NaCl, 4.7 mM KCl, 2.3 mM $CaCl_2$) and fixed in ethanol. The presence of sperm was observed via fluorescence microscopy after DAPI staining for nuclei was performed (Darrouzet *et al.* 2002).

To evaluate the number of secondary reproductives which had reproduced, we genotyped females and their offspring. Thus, for each *R. grassei* artificial colony of type 200 and 300, between 5 and 40 eggs or larvae from 1st to 3rd instar were collected with female neotenics. Offspring were frozen along with neotenic heads at -20°C.

Microsatellite analysis

Genotypes of *R grassei* neotenics and their offspring (eggs and larvae) were examined in the surviving artificial colonies: 6 of type 200, named 200-1 to 200-6 and 5 of type 300, named 300-1 to 300-5.

DNA was extracted from heads of neotenics, eggs and larvae by a Chelex® extraction method (Walsh *et al.* 1991). Samples were crushed, then mixed with 200 µl of a 5% Chelex® solution and 3 µl of a 1% proteinase K solution. After 1 hour of incubation at 56°C, samples were homogenized for 10 sec and placed at 96°C during 15 min. After a centrifugation at 8000 g for 3 min, 100 µl of the supernatant was removed and purified with a solution of absolute ethanol. We examined the microsatellite genotypes for 6 loci: Rf 6-1, Rf 5-10, Rf 21-1, Rf 11-1, Rf 11-2 and Rf 24-2 (Vargo 2000, DeHeer *et al.* 2005). PCR was conducted in a total volume of 10 µl, containing 3 mM or 1.5 mM MgCl2, 2 µM of reverse primer and 0.5 or 1 µM of forward primer, 0.04 units of *Taq* DNA polymerase and 1 µl genomic DNA. One primer of each pair was fluorescence-labelled. The PCR conditions were 40 cycles of 1min denaturation at 94°C, annealing at 57°C for 1min and elongation at 72°C for 15 sec. Then PCR products were denaturated at 94°C in a blue-bromophenol and formamide solution and run in a 6% denaturated polyacrylamide gel using a LiCor automated sequencer (1500 V). Microsatellite alleles were detected through their fluorescence and scored using the computer program GENE PROFILER 4.03 (Scanalytics, Inc.).

Individuals from each artificial colony were strongly related, thus the linkage disequilibrium and the deviations from Hardy-Weinberg equilibrium were analysed using a resampling method. A single individual per artificial colony was randomly chosen; a total of 20 data sets were created. Analyses were performed using the software GENEPOP v3.4 (Raymond & Rousset 1995). General descriptive statistics, such as observed versus expected heterozygosity were calculated for each artificial colony, using the software GDA (Lewis & Zaykin 2001).

To determine if one or several reproductives had reproduced we classified artificial colonies as simple or extended families. In simple families, offspring are produced by one pair of reproductives and observed offspring genotype frequencies did not differ from those expected under Mendelian segregation of alleles from two parents. In extended families, genotype frequencies within

colonies are not consistent with being produced by one pair of reproductives. Significance of the difference was determined by a G-test ($p<0.05$ when offspring were produced by more than two reproductives) (Bulmer *et al.* 2001, Goodisman & Crozier 2002, Vargo 2003, DeHeer & Vargo 2004).

Data analysis

The differences between the proportions of surviving workers, the number of neotenics and their offspring were tested using a Kruskall-Wallis test or a Mann-Whitney *U* test within *R. grassei* and *R. santonensis* artificial colonies of type 30, 50, 100, 200 and 300. To perform these tests, we made the assumption that all the females observed in the artificial colonies had mated and reproduced. Thus, the mean number of offspring (eggs and larvae) was estimated per female. All the analyses were performed with the software STATISTICA version 6 (StatSoft France, 2003).

RESULTS

Development of artificial colonies after 12 months

Survival

Twelve months after the beginning of the experiment, the number of artificial colonies with surviving termites was 27/30 for *R. grassei* and 23/30 for *R. santonensis*. Considering only the artificial colonies with surviving termites, the mean percentage of *R. grassei* workers initially placed in the box, defined as 'old' workers, varied from 48.5% to 76% between artificial colonies (Fig. 1). The mean percentages of 'old' workers in artificial colonies of *R. santonensis* were 17.1% to 44% (Fig. 1). For both species, the proportion of surviving 'old' workers was more important when the initial group size was small (Kruskall-Wallis test, $\alpha = 0.05$): $p = 0.006$ in *R. grassei* artificial colonies (n = 27) and $p = 0.031$ in *R. santonensis* artificial colonies (n = 23).

Development of nymphs and soldiers

In *R. grassei* artificial colonies, 1 or 2 nymphs were observed in 13 artificial colonies (table 1). In 5 *R. santonensis* artificial colonies (only type 200 and 300), 1 to 3 nymphs had differentiated (table 1). The presence of soldiers was first noticed between 3 or 4 weeks after we orphaned the artificial colonies. One soldier (or white soldier) was observed after 12 months in 4 artificial colonies of *R grassei* and in 3 artificial colonies of *R. santonensis* (Table 1).

Table 1. Number of nymphs and soldiers per artificial colony in *R. grassei* and *R. santonensis*. Number of artificial colonies with nymphs or soldiers are in brackets.

	R. grassei		*R. santonensis*	
Type	nymphs	soldiers	nymphs	soldiers
30	1, (2)			
50	1-2, (2)	1, (1)		
100	1, (3)	1, (1)		1, (1)
200	1, (1)		2-3, (2)	1, (2)
300	1-3, (5)	1, (2)	1-2, (3)	1, (1)

Because of the very small numbers of nymphs and soldiers produced in the artificial colonies, no statistical test could be performed to evaluate a difference related to the sizes of artificial colonies.

Development of neotenics and their offspring

Neotenics of *R grassei* and *R. santonensis* were first observed respectively 6 and 5 months after the beginning of the experiment. In the *R. grassei* artificial colonies, all the secondary reproductives observed were females. Their number ranged from 0 to 3 per artificial colony and increased with colony size (Kruskall-Wallis test, n = 27, $p = 0.000$) (Fig. 2). When we analyzed the percentage of female neotenics (per 100 workers), values obtained for *R. grassei* artificial colonies were significantly different among types (Kruskall-Wallis test, n = 27, $p = 0.001$). In *R. grassei* artificial colonies of type 100, 200 and 300, the mean offspring per female ranged from 82 to 211 eggs and larvae (Fig. 3). The mean offspring recorded in type 300 artificial

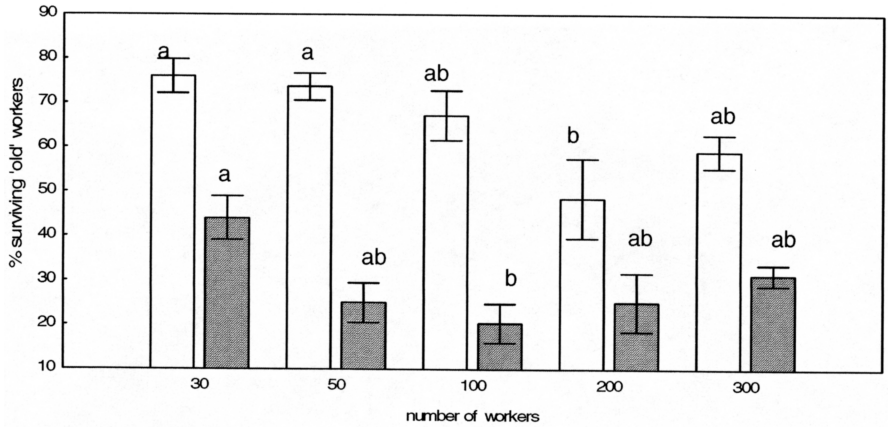

Fig. 1. Survival (mean ± SE) in *R. grassei* (white) and *R. santonensis* (grey) artificial colonies 12 months after orphaning. Different letters indicate significant differences within species ($p < 0.05$).

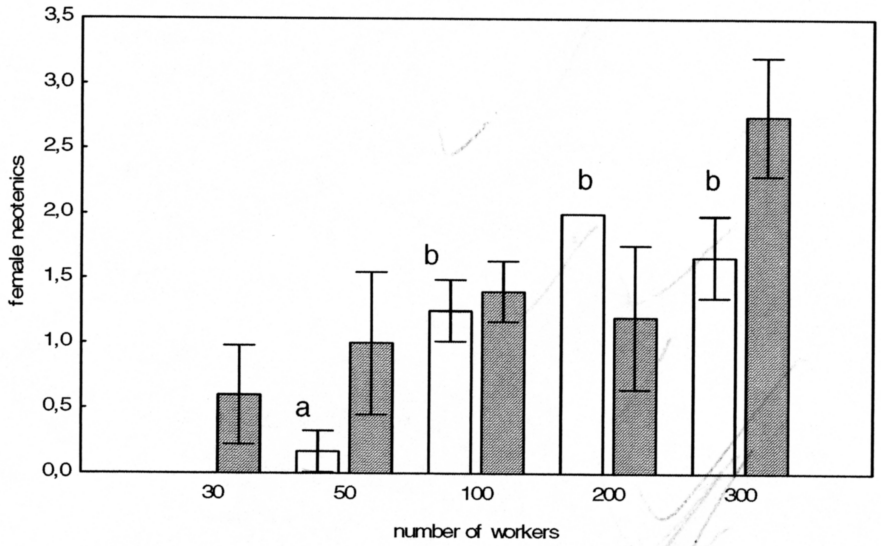

Fig. 2. Mean number (±SE) of *R. grassei* (white) and *R. santonensis* (grey) female neotenics per artificial colony, 12 months after orphaning. Different letters indicate significant differences within species ($p < 0.05$).

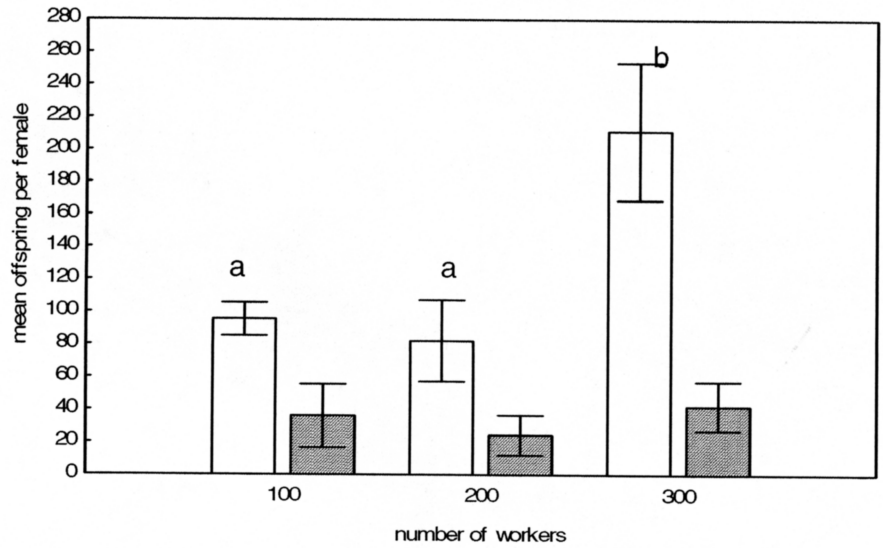

Fig. 3. Mean offspring per female (±SE) in *R. grassei* (white) and *R. santonensis* (grey) artificial colonies 12 months after orphaning. Different letters indicate significant differences within species ($p < 0.05$)

colonies was significantly higher than in type 100 and 200 artificial colonies (Mann-Whitney U test, $p < 0.05$).

In *R. santonensis* artificial colonies, the number of female neotenics ranged from 1 to 4 per colony and was not significantly different between types of artificial colonies (Kruskall-Wallis test, n = 23, $p > 0.05$) (Fig. 2). Male neotenics were also observed in one artificial colony of type 30 and in type 100, 200 and 300 artificial colonies (table 2). Male and female neotenics were observed in the same artificial colonies and their respective numbers were not different (Mann-Withney *U* test, p> 0.05). The mean offspring per *R. santonensis* female was between 24 and 50 eggs and larvae (Fig. 3).

Table 2. Mean number of female and male neotenics per artificial colony in *R. santonensis* series 12 months after orphaning.

Artificial colony size	Neotenics (females)	Neotenics (males)
30	1.5	0.5
50	2	0
100	1.4	1.5
200	2	2.67
300	2.75	1.5
Mean ± SE	1.93 ± 0.53	1.23 ± 1.03

Development of *R. grassei* artificial colonies of type 30, 50 and 100 and all *R. santonensis* artificial colonies after 32 months.

Survival

After 32 months of orphaning, the number of *R. grassei* artificial colonies with living termites was 5/6 in type 30, 6/6 in type 50 and 4/6 in type 100. The artificial colonies of type 30 and 50 had a similar proportion of 'old' workers (i.e. original workers): 34% and 38% respectively (Fig. 4). This percentage highly decreased in type 100 artificial colonies (6%).

The number of surviving artificial colonies of *R. santonensis* was very low. Only 9/30 artificial colonies had living termites, all from type 30 and 50. The mean percentage of 'old'workers within these artificial colonies was 44 and 25% respectively (Fig. 4).

Development of nymphs and soldiers

The development of nymphs and soldiers occurred only in *R. grassei* artificial colonies (type 30, 50 and 100). One or two nymphs and one white soldier were observed in two artificial colonies of type 30. Two artificial colonies of type 100 had one and five nymphs.

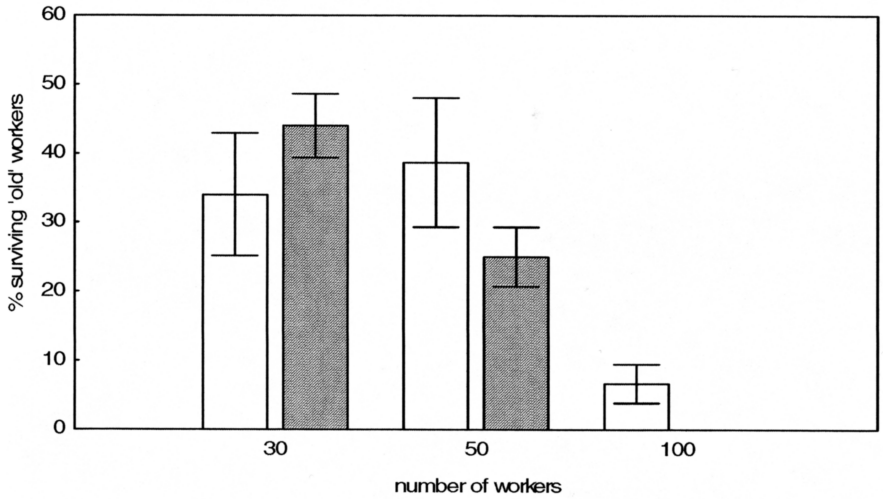

Fig. 4. Survival (mean ± SE) in *R. grassei* (white) and *R. santonensis* (grey) artificial colonies 32 months after orphaning.

Development of neotenics and their offspring

The *R. grassei* type 30, 50 and 100 artificial colonies were able to support the differentiation of neotenics and the production of their offspring. In three type 30 and type 50 artificial colonies, one or two female neotenics and one male neotenic per artificial colony were recorded. The four type 100 termite artificial colonies had a mean number of 1.25 males and 2.5 females. The numbers of neotenics were not significantly different among the types and the number of females was not significantly higher than the number of males (Kruskall-Wallis test, n = 4, $p > 0.05$). The mean offspring (eggs and larvae) per female was between 2 and 98 individuals, but brood size was not related to the original size of the artificial colonies (Kruskall-Wallis test, n = 10, $p>0.05$).

In *R. santonensis* groups, 1 or 2 female neotenics were observed in 3 type 50 artificial colonies and the brood production was much reduced, around 12 larvae per artificial colony.

Development of female and male neotenics in *R. grassei* type 200 and 300 artificial colonies.

Twenty-four months after the beginning of the experiment, we tried to observe male neotenics in the artificial colonies of *R. grassei* with female

neotenics. Only one artificial colony (type 200) contained 3 male neotenics. The offspring production in the other artificial colonies could occur through female parthenogenesis, as observed in orphaned colonies of *R. speratus* (Kolbe) (Hayashi *et al.* 2003, Hayashi *et al.* 2006). Male neotenics were present but they did not have the morphological characteristics usually observed on neotenics. Females from type 200 and 300 artificial colonies were dissected. Within type 200 artificial colonies, 11 female neotenics were observed and dissected. Among them, 7 had mature eggs in variable quantities. Due to a technical problem during dissections we were able to dissect the spermathecae of 9 females (instead of 11) and 6 of them contained stored sperm at the time we took them from the artificial colonies. Within the type 300 artificial colonies, we identified and dissected 10 female neotenics; five of them had mature eggs. During dissections of spermathecae, one was damaged. Thus spermathecae of 9 females were dissected and we observed that all stored sperm. We could conclude from these observations that in type 200 and 300 artificial colonies of *R. grassei*, several neotenic females had mated with neotenic males, but some females did not produce eggs at the time they were isolated.

Genotyping

In *R. grassei* artificial colonies of type 200 and 300, only 2 loci were found to be polymorphic: Rf6-1 and Rf21-1, with 2 alleles each. We scored between 1 and 4 neotenics and between 5 and 40 eggs or larvae from each artificial colony. The resampled data sets showed that none of the two loci were in linkage disequilibrium or had shown deviations from the Hardy-Weinberg equilibrium.

In some artificial colonies (for the locus Rf6-1, colony 200-5 as example), genotypes of female neotenics were homozygous and some of their offspring were heterozygotes. From this observation we confirmed the reproduction of females with male neotenics.

In 83.3% of type 200 artificial colonies, the offspring was produced by a single pair of reproductives (simple families, G-test $p > 0.05$) (Table 3). The proportion of simple families is 60% in type 300 artificial colonies. In a majority of *R. grassei* artificial colonies, more than one female neotenics were observed but all females did not reproduce.

DISCUSSION

From previous studies, we know that a *Reticulitermes* colony can comprise from 50000 up to 1 million individuals in natural and urban habitats (Howard *et al.* 1982; Paulmier *et al.* 1997). The presence of *R. grassei* and *R. santonensis* in urban habitats is certainly related to anthropic factors. Only a fraction of a colony could be introduced through the transport of wood or plants with a lump of soil and then invade an entire area. Studies on the breeding system of these species in urban environment showed that all *R. santonensis* colonies and 51.8% of *R. grassei* colonies were headed by multiple secondary reproductives (Dronnet *et al.* 2005; DeHeer *et al.* 2005).

Nests of *Reticulitermes* species are subterranean and foragers collect the cellulose in log-woods situated on the ground. Foragers can adjust their food uptake to the characteristics of the colony (numerous dependant individuals); food quantity and quality can have an effect on the caste composition of a colony (Lenz 1994). In our experimental design, a new piece of poplar wood was supplied regularly. We considered that food resource, temperature and relative humidity were not limiting factors to the development of artificial colonies, as in human housing.

From our preliminary results, we observed that under laboratory conditions, a group of 30 workers can survive over a long period (24 months) and produce secondary reproductives and a new generation of termites. The colony

Table 3. Numbers (N) of neotenics, eggs and larvae genotyped, observed (Ho) and expected (He) heterozygosities, structure of the family obtained by G-test ($p< 0.05$: extended family) in each *R. grassei* artificial colony of type D (200 termites) and E (300 termites).

Colony	N	He neotenics	Ho neotenics	N (eggs)	N (larvae)	He offspring	Ho offspring	Family type
200-1	2	0.250	0.250	10		0.252	0.278	simple
200-2	2	0.250	0.250	20	4	0.473	0.595	simple
200-3	4	0.428	0.500	20	1	0.198	0.164	extended
200-4	2	0.250	0.250	10	1	0.331	0.394	simple
200-5	2	0.250	0.250	5		0.278	0.300	simple
200-6	2	0.583	0.750	40		0.507	0.396	simple
300-1	2	0.500	0.500		20	0.328	0.418	extended
300-2	3	0.381	0.417	2	25	0.326	0.315	extended
300-3	3	0.333	0.333		25	0.333	0.411	simple
300-4	1	-	-		12	0.475	0.633	simple
300-5	3	0.167	0.167		20	0.378	0.485	simple

growth is relatively slow but it could be sufficient to invade an urban habitation within few years. Some authors consider that a group of 50 termites is sufficient to establish a colony (Serment & Tourteaux 1991).

The survival of the *R. santonensis* orphaned colonies was significantly low. This result is in contrast with observations made frequently on several field colonies of *R. santonensis*. The high mortality within artificial colonies could be due to the preservation of the colony in laboratory conditions during a long period, and thus, to a decrease of its strength. However, in both species, a majority of the artificial colonies were active twelve months after the start of the experiment and several differences were observed among different sizes of artificial colonies. The workers of artificial colonies with small sizes seemed to survive better after 12 months than artificial colonies with 200 or 300 termites. Considering all the plastic boxes used in the experiment had the same volume, the density of individuals was variable and could have an influence on the mortality of colony members.

In their natural habitat, *Reticulitermes* soldiers usually represent between 1 and 3% of the colony members, although higher values have been reported (Grace 1996; Forschler & Jenkins 1999; Long *et al.* 2003). In the present study, a maximum number of one soldier per artificial colony was observed, whatever the colony size was. They appeared within 3 or 4 weeks after the orphaning. The development time from a worker to a soldier is usually longer than 4 weeks (Büchli 1958; Lainé & Wright 2003). Therefore, we can suppose that some workers had initiated their differentiation in soldiers before the orphaning happened. In a similar study on orphaned colonies of *R. hageni*, *R. virginicus* and *R. flavipes*, Pawson and Gold (1996) have recorded up to 4 soldiers per colony and their number was increasing with worker density. However, the low number of soldiers in our experiment could be explained by the local conditions: stable temperature and humidity, lack of predators and/or competitors. Moreover, the differentiation of dependant individuals as soldiers could happen at the expense of colony growth.

A small number of nymphs were observed 12 months after orphaning. Nymphs are immature and dependent individuals; they can differentiate into adults (primary reproductives) or into nymphoid neotenics (secondary reproductives). Miyata *et al.* (2004) have observed in a study on the pattern of neotenic differentiation in *R. speratus* that ergatoid differentiation required

a longer time than nymphoid differentiation. Büchli (1958) hypothesized that the differentiation of *Reticulitermes* workers into neotenics was difficult because workers could not accumulate enough resources to have a rapid differentiation. In our experiment, these nymphs would probably differentiate into neotenics.

We observed male neotenics of *R. grassei* that were not morphologically distinguishable from a worker or a nymph several months after the orphaning. Thus, male neotenics could be sexually mature and be able to inseminate females before the external morphological characteristics, used generally to identify male neotenics (darker pigmentation, longer abdomen, presence of eyes, etc.), appear. Further studies are needed to confirm the presence of what could be named 'hidden' male neotenics.

The number of female neotenics observed in the artificial colonies, after the orphaning, was variable: from 1 up to 4 per colony. These values were similar to the number of reproductives produced by *R. speratus* orphaned colonies (Watanabe & Noda 1991) but lower than the number of secondary reproductives observed in *R. virginicus* or *R. flavipes* orphaned colonies (Pawson & Gold 1996). More reproductives had differentiated at high densities of workers than at low densities. Similar results were found with orphaned colonies of *R. hageni*, *R. virginicus* and *R. flavipes* (Pawson & Gold 1996).

From several studies, it was assumed that numerous replacement reproductives have a greater egg production than the primary queen (Miller 1969; Nutting 1970; Myles 1999). However, several authors consider that the queen fecundity was superior to those of neotenics (Noirot 1990; Thorne 1996, 1998; Lainé & Wright 2003). In our study, the female fecundity was variable among artificial colonies. Nevertheless, the values estimated were in the same order than found by Pawson & Gold (1996).

We tried to assess the number of reproducing females in some *R. grassei* artificial colonies. The loci tested were not highly polymorphic. However, we performed G-tests to determine if the brood was produced by one parental pair or by several parents. Simple families (one pair of reproductives) occurred in half of the artificial colonies. Colonies could be headed by the female neotenic who first differentiated during the experiment, which could dominate the sexual development of other females. The other female neotenics could be differentiated but their ovocyte maturation could be delayed. Dissections

revealed that several female neotenics did not have mature ovocytes. In other artificial colonies, the brood was produced by several reproductives but the exact number of females reproducing could not be evaluated. Further studies with more polymorphic colonies could determine the exact number of females producing the new generation and thus, the mean fecundity could be evaluated more precisely. Moreover, other experiments could investigate the mechanisms of a possible dominance of one female neotenic (whether the first to differentiate or not).

As we chose to collect the first results after one year of the experiment, so as not to disturb the colonies too frequently, we did not obtain results on the egg and larval development times or on the aggression between workers and newly differentiated neotenics or among reproductives, as studied previously in other termite species (Lenz & Barrett 1982; Lenz & Runko 1993; Roisin 1993, 2000).

Because of the low number of colonies used in this first study, we could not evaluate the variation of caste differentiation and reproduction strategies within and among the two species. However, termites of the genus *Reticulitermes* have flexible developmental pathways which allow the persistence of colonies in absence of primary reproductives. A single pair of secondary reproductives in a colony with 100 workers can produce a new generation. Therefore a special attention must be paid to the human-mediated transport of termites and to the control strategies of fractionate colonies.

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