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In the present study, trail pheromone blends are identified for the first time in termites. In the phylogenetically complex Nasutitermitinae, trail-following pheromones are composed of dodecatrienol and neocembrene, the proportions of which vary according to species, although neocembrene is always more abundant than dodecatrienol (by 25–250-fold). Depending on species, termites were more sensitive to dodecatrienol or to neocembrene but the association of both components always elicited significantly higher trail following, with a clear synergistic effect in most of the studied species. A third component, trinervitatriene, was identified in the sternal gland secretion of several species, but its function remains unknown. The secretion of trail pheromone blends appears to be an important step in the evolution of chemical communication in termites. The pheromone optimizes foraging, and promotes their ecological success. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, **99**, 20–27.

ADDITIONAL KEYWORDS: (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol – neocembrene – species-specificity – trinervitatriene.

INTRODUCTION

In the context of a study on the evolution of chemical communication in termites, trail pheromones were investigated in the phylogenetically most advanced termite group, the Nasutitermitinae.

Although the pheromones of insects are most often composed of pheromonal blends, especially in social Hymenoptera (Billen & Morgan, 1998; Wyatt, 2003), until now, only individual components of trail pheromone blends have been identified in termites (Pasteels & Bordereau, 1998; Bordereau & Pasteels, 2009) where these pheromones have a unique glandular origin, the sternal gland (Noirot, 1969; Quennedey *et al.*, 2008).

However, the observation of species-specific trail following strongly suggested multi-component trail-following pheromones made from a common component eliciting moving, together with minor components bringing species-specificity in termites (Howard, Matsumura & Coppel, 1976; Kaib, Bruinsma & Leuthold, 1982; Peppuy *et al.*, 2001a, b). Traniello (1982), Hall & Traniello (1985) in *Nasutitermes corniger* (formerly *Nasutitermes costalis*), and Runcie (1987) in *Reticulitermes flavipes* claimed the existence of an ephemeral volatile component for recruitment and a long-lasting component for

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Table 1. List of studied species, with their geographical distribution, the place of collection, and the place of bioassays

	Distribution	Collection	Bioassays
Constrictotermes cyphergaster (Silvestri)	Neotropical	Brazil	Brazil
Nasutitermes corniger (Motschulsky)	Neotropical	Guadelupe	France
Nasutitermes ephratae (Holmgren)	Neotropical	Guadelupe	France
Nasutitermes guayanae (Holmgren)	Neotropical	French Guiana	France
Nasutitermes kemneri Snyder & Emerson	Neotropical	Brazil	Brazil
Nasutitermes lujae (Wasmann)	Aethiopian	Ivory Coast	France
Nasutitermes voeltzkowi (Wasmann)	Malagasy	Mauritius	France
Nasutitermes exitiosus (Hill)	Australian	Australia	Australia
Trinervitermes geminatus (Wasmann)	Aethiopian	Ivory Coast	France
Trinervitermes trinervoides (Sjöstedt)	Aethiopian	South Africa	France

orientation. At the surface of the sternal gland of workers of *Macrotermes annandalei* and *Macrotermes barneyi*, Peppuy *et al.* (2001a, b) identified several specific compounds besides the common one for orientation, but were unable to identify their function. Finally, in several species, the estimated amount of the trail-following pheromone did not correspond well with the biological activity of sternal gland extracts, suggesting subsidiary components acting in synergy (Laduguie *et al.*, 1994; Sillam-Dussès *et al.*, 2005). Nevertheless, until now, no minor components have been identified in these trail pheromone blends, although electroantennography (EAG) and gas chromatography and electroantennographic detection (GC-EAD) experiments strongly suggested (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (dodecatrienol) as a minor component of the trail pheromone of *Prorhinotermes simplex* (Sillam-Dussès *et al.*, 2009a).

Using the method of solid phase microextraction (SPME) that allows specific extraction of the glandular secretions, we re-investigated the Australian termite, *Nasutitermes exitiosus* where neocembrene was identified as a trail-following pheromone (Birch *et al.*, 1972), and extended this study to nine other species of Nasutitermitinae of different geographic origins (Table 1) with the aim of analysing the chemical evolution of trail pheromones in termites. The name Nasutitermitinae refers here to full nasutes (Engel & Krishna, 2004), which are considered as the most phylogenetically advanced termites (Inward, Vogler & Eggleton, 2007).

MATERIAL AND METHODS

Chemical analysis

Chemical analyses were performed in Dijon (France). The method consisted of comparing by GC-mass spectrometry (MS) the compounds extracted after SPME from the surface of the sternal gland and from the surface of the nonglandular integumental areas of workers. This allowed us to isolate the compounds specific to the sternal gland secretion. According to the size of termites and the activity of the sternal gland, extracts from 20–50 workers were necessary for a GC-MS analysis (Table 2). SPME and GC-MS analyses were performed as previously described by Peppuy *et al.* (2001b) and Sillam-Dussès *et al.* (2007).

Standards

Synthetic dodecatrienol was synthesized by the Wood Research Institute of Kyoto University. Neocembrene and trinervitatriene were purified from the tergal glands of alate females of *Nasutitermes voeltzkowi* and *Nasutitermes ephratae*, respectively (purity degree = 98%) (Sillam-Dussès *et al.*, 2005 and Buděšínský *et al.*, 2005).

Bioassays

Trail-following bioassays were carried out as described by Sillam-Dussès *et al.* (2009b) with freshly-collected termites in the country of collection or with healthy termites maintained under constant climatic conditions in France (temperature = 26 °C, relative humidity = 80%) (Table 1). Because of the short survival period of tropical termites, which could not be brought back to France within their whole nests, all of the bioassays could not be performed for all species. Choice tests for species-specificity were analysed with a Kruskall–Wallis test, whereas a Mann–Whitney test was performed to evaluate synergistic activity after an arbitrary sum of the data of each factor.

RESULTS

Chemical data

Nasutitermes exitiosus as an example

GC profiles of sternal gland extracts in comparison to nonglandular cuticular extracts revealed the

Table 2. Chemical nature of trail-following pheromones

	N	Dodecatrienol	Neocembrene	Ratio D/N	Trinervitatriene
Constrictotermes cyphergaster	40	+	+	ND	–
Nasutitermes corniger	40	+	+	1/25	+
Nasutitermes ephratae	40	+	+	1/25	+ (traces)
Nasutitermes guayanae	40	+	+	1/250	–
Nasutitermes kemneri	40	+	+	1/25	–
Nasutitermes lujae	20	+	+	1/25	–
Nasutitermes voeltzkowi	20	+	+	1/25	+ (traces)
Nasutitermes exitiosus	50	+	+	1/70-100	–
Trinervitermes geminatus	50	+	+	1/100	–
Trinervitermes trinervoides	50	+	+	1/25	–

A SPME fiber was rubbed on the sternal gland of workers and then was desorbed by gas chromatography-mass spectrometry to identify the compounds (*N*, number of individuals analysed; D/N, ratio dodecatrienol : neocembrene; ND, not determined).

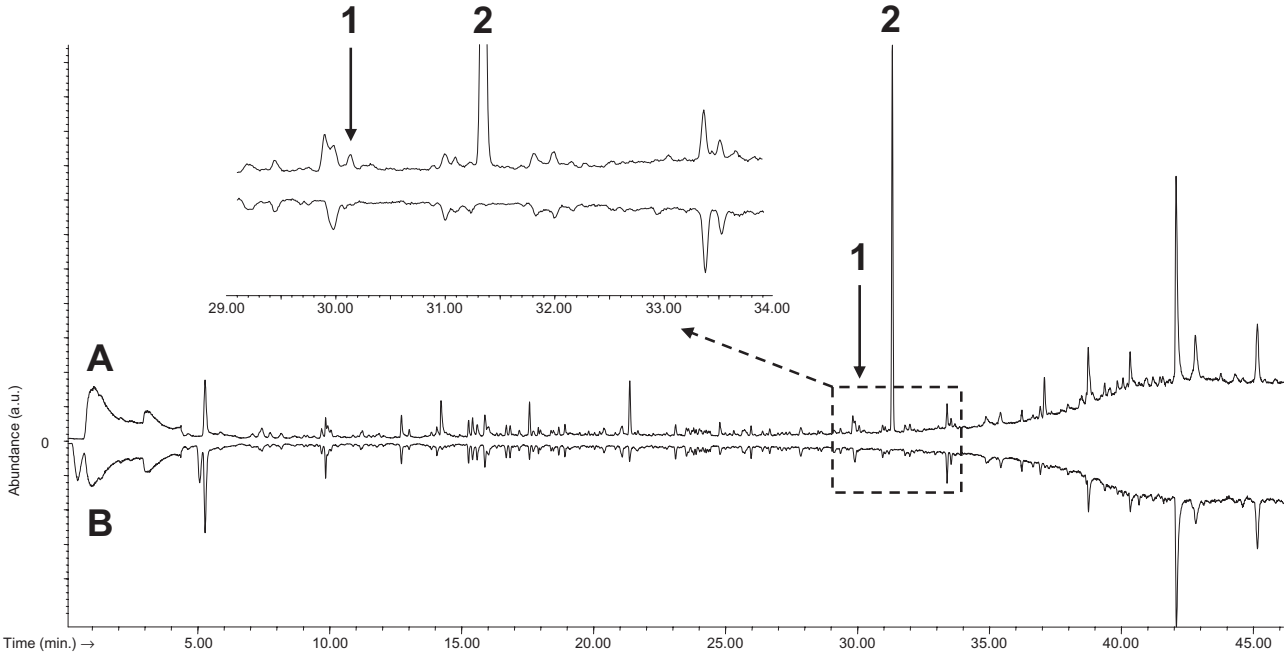

Figure 1. Gas chromatography profiles on a DB-wax column of solid phase microextraction extracts of the sternal gland surface (A) and the abdominal tergal surface (B) of workers of *Nasutitermes exitiosus* showing two compounds specific to the sternal gland secretion, dodecatrienol (1) and neocembrene (2). Additional detail is provided in the inset.

presence of numerous common compounds (essentially cuticular hydrocarbons) and only two compounds specific to the sternal gland (Fig. 1). A few peaks were also only present on the GC profile of either the sternal gland surface or the tergal surface, but MS showed they were artefacts coming from the phase covering the SPME fibre. Retention indices on polar (DB-wax) and apolar (VF5-ms) columns of the two compounds specific to the sternal gland were LRI 2190 and LRI 1498, respectively, for the most volatile component, and LRI 2260 and LRI 1957, respectively, for the less volatile one. These values and the EI mass spectral characteristics correspond, respectively, to the standards of dodecatrienol, and neocembrene (for reference LRIs and EI mass spectra, see Bordereau *et al.*, 1991 and Sillam-Dussès *et al.*, 2005). In this Australian species, the amount of dodecatrienol was estimated at 10–15 pg per worker and the amount of neocembrene at 1 ng per worker (ratio = 1 : 70–100).

Other Nasutitermitinae

The two compounds were identified in all Nasutitermitinae studied. Neocembrene was always more abundant than dodecatrienol, although the relative proportions varied according to species, with the highest detectable ratio for dodecatrienol/neocembrene reaching 1 : 250 in *N. guayanae*. In *Constrictotermes cyphergaster*, *Nasutitermes guayanae*, *Nasutitermes lujae*, and *Trinervitermes geminatus*, dodecatrienol was only present at trace levels (Table 2). SPME is not an accurate quantitative technique of extraction; therefore, the ratios are best given as an order of magnitude. In *N. corniger*, besides dodecatrienol and neocembrene, trinervitatriene (LRI 2290 on a DB-wax column, mass spectrum as in Buděšínský *et al.*, 2005) also was specifically present on the sternal gland surface (trinervitatriene/neocembrene ratio = 1 : 2). Traces of trinervitatriene (ratio with neocembrene = 1 : 50) were also found in *N. ephratae* and *N. voeltzkowi*.

Biological activity

When tested alone, dodecatrienol or the neocembrene did not elicit high trail following at low concentrations (Table 3). With dodecatrienol, the activity threshold was generally barely obtained with 10^{-3} and 10^{-2} ng cm^{-1}, except for *N. corniger*, in which workers did not follow trails made of dodecatrienol at any concentration. Workers of *N. voeltzkowi* appeared to be the most sensitive to dodecatrienol (activity threshold obtained at a concentration as low as 5.10^{-4} ng cm^{-1}), but good trail followings were also obtained with *Trinervitermes trinervoides*, and to a lesser degree with *T. geminatus*, *N. ephratae*, and *C. cyphergaster*.

With neocembrene, the trail-following threshold was obtained at 10^{-2} ng cm^{-1} for *N. voeltzkowi*, but only at 10^{-1} ng cm^{-1} for most other tested species, and at 1 ng cm^{-1} for *N. kemneri*. Trail following was clearly better with neocembrene than with dodecatrienol for *N. corniger*, *N. ephratae*, and especially *N. exitiosus* and *N. voeltzkowi*, but, nevertheless, was never maximal (10 cm).

By contrast, trail following was significantly better on trails made of a mixture of dodecatrienol and neocembrene than on trails made of these compounds individually at the same concentration (Table 4). For example, workers of *T. geminatus* followed trails comprising a mixture of dodecatrienol at 10^{-2} ng cm^{-1} and neocembrene at 1 ng cm^{-1} (9.8 ± 0.2 cm) better than trails comprising only dodecatrienol or neocembrene. When given a choice between a trail made of this mixture of dodecatrienol and neocembrene and a trail made of sternal gland extract (1 sternal gland cm^{-1}), workers of *T. geminatus* did not show a statistically

Table 3. Trail-following bioassays

	Dodecatrienol						Neocembrene			
Concentration (ng cm^{-1})	5×10^{-4}	10^{-3}	5×10^{-3}	10^{-2}	10^{-1}	1	10^{-3}	10^{-2}	10^{-1}	1
Constrictotermes cyphergaster	–	1.8 ± 0.6	–	4.2 ± 1.3	2.5 ± 0.5	1.9 ± 0.4	0.3 ± 0.1	2.3 ± 0.9	3.4 ± 1.2	1.3 ± 0.4
Nasutitermes corniger	–	1.5 ± 0.4	1.2 ± 0.2	2.1 ± 0.4	1.9 ± 0.4	1.9 ± 0.6	0.5 ± 0.2	2.6 ± 0.5	5.6 ± 0.7	1.1 ± 0.4
Nasutitermes ephratae	–	1.4 ± 0.3	4.5 ± 0.6	4.9 ± 0.7	2.0 ± 0.5	2.6 ± 0.6	1.1 ± 0.3	2.3 ± 0.5	6.2 ± 0.7	3.5 ± 0.6
Nasutitermes exitiosus	–	2.2 ± 0.4	–	3.2 ± 0.5	0.9 ± 0.3	1.6 ± 0.2	0.5 ± 0.2	1.2 ± 0.2	4.3 ± 0.7	6.6 ± 0.7
Nasutitermes kemneri	–	3.5 ± 1.2	–	0.9 ± 0.3	0.4 ± 0.4	–	1.1 ± 0.3	1.0 ± 0.3	0.8 ± 0.3	3.5 ± 0.8
Nasutitermes voeltzkowi	4.7 ± 0.7	5.8 ± 0.7	4.0 ± 0.9	2.3 ± 0.6	–	–	1.5 ± 0.3	2.4 ± 0.4	7.2 ± 0.6	8.8 ± 0.5
Trinervitermes geminatus	–	1.7 ± 0.4	–	5.1 ± 0.7	2.6 ± 0.5	0.9 ± 0.2	1.4 ± 0.4	2.7 ± 0.6	5.7 ± 0.7	2.8 ± 0.5
Trinervitermes trinervoides	–	1.9 ± 0.3	–	7.4 ± 0.6	8.7 ± 0.5	5.9 ± 0.6	0.9 ± 0.2	0.9 ± 0.3	1.2 ± 0.3	–

Values are distances of open-field trail following (mean ± SD in cm, $N = 30$) on 10-cm long artificial trails made of dodecatrienol or neocembrene at different concentrations.

Table 4. Trail-following bioassays

Tested species	Dodecatrienol			Neocembrene			D + N	Ratio D/N	*P*
	5×10^{-4}	5×10^{-3}	10^{-2}	10^{-2}	10^{-1}	1			
Trinervitermes geminatus	–	–	5.1 ± 0.7	–	–	2.8 ± 0.5	9.8 ± 0.2	1/100	< 0.001
Nasutitermes corniger	–	1.2 ± 0.2	–	–	5.6 ± 0.7	–	9.6 ± 0.7	1/20	< 0.001
Nasutitermes ephratae	–	4.5 ± 0.6	–	–	6.2 ± 0.7	–	9.8 ± 0.2	1/20	NS
Nasutitermes voeltzkowi	4.7 ± 0.7	–	–	2.4 ± 0.4	–	–	9.3 ± 0.4	1/20	< 0.001
Nasutitermes exitiosus	–	–	3.2 ± 0.5	–	–	6.6 ± 0.7	9.7 ± 0.3	1/100	NS

Values are distances of open-field trail following (mean ± SD in cm, $N = 30$) on 10-cm long artificial trails made of dodecatrienol (D), neocembrene (N), or a mixture of both. Trail following made with the mixture was significantly better than the sum of the trails made of only dodecatrienol and only neocembrene at the same concentration in all species except *N. ephratae* and *N. exitiosus* (Mann–Whitney test, NS, nonsignificant, $P < 0.001$). Dodecatrienol concentrations were chosen according to the activity threshold and the corresponding neocembrene concentrations were chosen according to the determined ratios. These concentrations reflected approximately the ratio determined for the compounds (ratio D/N = ratio dodecatrienol : neocembrene).

significant preference (17/13, $N = 30$). It must be noted that the third specific compound, trinervitatriene, identified at the surface of the worker sternal gland of *N. corniger*, *N. ephratae*, and *N. voeltzkowi*, was not available at the time of trail-following bioassays and could not be tested.

The mixture of dodecatrienol and neocembrene enhanced trail following in every species ($P < 0.001$, $N = 30$), and we observed a synergistic effect in *T. geminatus*, *N. corniger*, and *N. voeltzkowi* (Table 4). In *N. ephratae* and *N. exitiosus*, this synergistic effect probably also exists, but cannot be statistically demonstrated because of the relatively high trail-following responses obtained with only neocembrene and the conditions of the bioassay (trails of 10 cm in length).

Species-specificity of trail-following pheromones

Choice trail-following bioassays were performed to test the potential species-specificity of trail-following pheromones. Under our experimental conditions (i.e. open field trail following, trails made of sternal gland extracts), workers of *N. corniger* showed a preference for their own trails, whereas workers of *N. ephratae* did not preferentially follow their own trails. In another series of tests with *N. guayanae* and *N. voeltzkowi*, trail-following responses were statistically different and species-specific (Table 5).

DISCUSSION

In the present study, trail pheromone blends have been isolated and identified in phylogenetically advanced termites. In the ten species of Nasutitermitinae studied, trail pheromone blends are composed of dodecatrienol and neocembrene, with the proportions varying according to species, although neocembrene is always more abundant than dodecatrienol. Bioassays showed that only the association of the two components elicited trail following in workers similar to the trail following obtained using sternal gland extracts. When tested in mixture, dodecatrienol and neocembrene possessed a synergistic effect in three of the studied species. The third sternal gland-specific compound, trinervitatriene, identified in *N. corniger*, *N. ephratae*, and *N. voeltzkowi*, has yet to be tested for its behavioural effect.

Neocembrene was previously shown to be highly active in eliciting trail following and was therefore considered as being the only trail pheromone of the Australian termite *N. exitiosus* (Moore, 1966; Birch *et al.*, 1972), and the African termite *Trinervitermes bettonianus* (McDowell & Oloo, 1984). The present study shows that a mixture of neocembrene and dode-

Table 5. Choice trail-following bioassays

Tested species	Trail	Trail	*P*
	Nasutitermes corniger	*Nasutitermes ephratae*	
Nasutitermes corniger	12	3	**
Nasutitermes ephratae	6	9	NS
	Nasutitermes guayanae	*Nasutitermes voeltzkowi*	
Nasutitermes guayanae	13	2	***
Nasutitermes voeltzkowi	0	15	***

Values are choice for one of the trail. Trail-following pheromones appear species-specific. All trails were made of sternal gland extracts at 10^{-1} gland cm^{-1} (**$P < 0.01$, ***$P < 0.001$, NS, nonsignificant).

catrienol improves trail following in *N. exitiosus*, *N. ephratae*, *N. corniger*, *N. voeltzkowi*, and *T. geminatus*, and a synergistic effect of components could be demonstrated for the latter three species. In the neotropical *N. corniger* used as a model for the first studies on the trail pheromone in termites (Stuart, 1961, 1963), Hall & Traniello (1985) claimed that neocembrene, highly active in eliciting orientation, could only be one of the components of a trail pheromone blend. We show that it comprises a blend of at least neocembrene and dodecatrienol, and possibly of trinervitatriene as well. By contrast, other species secreting dodecatrienol and neocembrene at the surface of their sternal gland appear to be highly sensitive to dodecatrienol. This is the case for *N. voeltzkowi* (present study) and *N. lujae* (Laduguie, 1993), and, to a lesser degree for *T. trinervoides*. We could therefore postulate the existence of a gradient of sensitivity toward dodecatrienol or neocembrene according to species, with significant impacts on behaviour and species isolation.

The presence of trail pheromone blends, until now found only in the most advanced and ecologically successful termites, the Nasutitermitinae (present study), and in the Prorhinotermitinae (Sillam-Dussès *et al.*, 2009a), raises questions about the possible advantages of these pheromone blends. There is a possible increase in their biological activity as a result of lowering the activity threshold. This would allow a decrease in the metabolic cost of synthesis in termites that are generally of a small size and live in populous colonies. The longevity of the trail-following pheromone might be also increased: the neocembrene reducing the evaporation of the more volatile and less stable molecule of dodecatrienol. Most of the Nasutitermitinae are of the 'separate ecological life type' (Abe, 1987); hence, their workers, which forage far from the nest, might require long-lasting orientation signals.

We cannot exclude separate behavioural effects for the two components. In *N. corniger*, Traniello (1982) and Hall & Traniello (1985) highlighted the recruitment and orientation behaviours induced by pheromone exposure and postulated that they were a result of an ephemeral volatile compound and a long-lasting compound, respectively. We could therefore ascribe the role of recruitment to the more volatile component dodecatrienol, and the role of orientation to neocembrene. However, neocembrene most likely cannot remain active for many months or several years, as observed for trails of *N. corniger* (Traniello, 1982). Furthermore, dodecatrienol and neocembrene themselves were shown to individually possess both behavioural effects (recruitment and orientation), respectively, in *Reticulitermes santonensis* (Laduguie *et al.*, 1994) and in *Prorhinotermes canalifrons* and *P. simplex* (Sillam-Dussès *et al.*, 2005). Moreover, long-lasting trails also exist in the American species *R. flavipes* (Runcie, 1987), which is the same species as the European *R. santonensis* (Austin *et al.*, 2005), where the trail-following pheromone is only composed of dodecatrienol (Laduguie *et al.*, 1994).

As recently shown by Jackson *et al.* (2007) for ant foraging trails, multi-component pheromones allow for high behavioural sophistication. Trail pheromone blends with possible caste-specific proportions of compounds could partly explain the complex and highly-regulated foraging polyethism observed in Nasutitermitinae (Pasteels, 1965; Traniello, 1981; Traniello & Busher, 1985; Laduguie, 1993; Traniello & Leuthold, 2000). Finally, the trail pheromone blends of Nasutitermitinae may be involved in species-specificity of trails, which would optimize foraging by delineating territories and limiting conflicts. This could be the result of either species-specific components, or species-specific ratios between common components. For example, in *N. corniger* and *N. ephratae*, which possess similar ratios of dodecatrienol/neocembrene, the species-specific responses of *N. corniger* workers could be a result of trinervitatriene, which is present in much higher amounts in the sternal gland secretion of this species. However, *N. ephratae* workers that do not exhibit species-specific trail following would follow dodecatrienol and

neocembrene present in both species. By contrast, in the case of *N. guayanae* and *N. voeltzkowi*, the dodecatrienol/neocembrene ratio is quite different and trail-following responses were entirely species-specific. Nevertheless, traces of trinervitatriene in *N. voeltzkowi* might also explain these species-specific responses.

In summary, Nasutitermitinae, which are considered to be the most phylogenetically advanced termites (Inward *et al.*, 2007; Legendre *et al.*, 2008), are known for their impressive ecological success in many tropical areas of the ethiopian and the neotropical zones. Trail pheromone blends apparently lead to the fine-tuning of behaviours and species-specific properties, and hence optimize foraging, which might be a key factor explaining their success.

The chemical evolution of trail-following pheromones is remarkably conservative in termites where dodecatrienol and/or neocembrene are known to be major components of the trail-following pheromones in many taxa of all subfamilies of Rhinotermitidae, as well as in Termitidae, which represent more than three-quarters of the termite species (Bordereau & Pasteels, 2009). This remarkable simplicity of termite trail pheromones, compared to the high diversity of ant trail pheromones (Billen & Morgan, 1998; Jackson *et al.*, 2007) might be due to the uniqueness of their glandular source, the sternal gland (Noirot, 1969), and to the simplicity of the food source of termites.

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