Endogenous synchronization of the chemical signature of *Reticulitermes* (Isoptera: Rhinotermitidae) worker termites

Anne-Geneviève Bagnères, Eric Darrouzet, Xavier Landré & Jean-Philippe Christidès

Institut de Recherche sur la Biologie de l'Insecte, UMR CNRS 6035, Université François Rabelais de Tours, Faculté des Sciences et Techniques,
Parc de Grandmont, F-37200 Tours, France

Abstract. Termites of the genus *Reticulitermes* are characteristic of temperate regions. Their colonies comprise various castes, the most numerous being that of workers which can develop into soldiers or secondary reproductives (neotenics). Each caste has a mixture of hydrocarbons (HCs) on the cuticle forming a chemical signature. The primary aim of this study was to compare the changes in the chemical signature of a population of worker termites fed on paper with juvenile hormone to differentiate them into soldiers with a control population of termites fed only on paper or wood for one month. Gas chromatography was used to analyze the cuticular profiles of *Reticulitermes flavipes* termites to determine whether they changed, and, if so, when and how. The data collected over one month showed that the workers fed with JH did not differentiate into soldiers but that there were progressive changes in the hydrocarbon profile independent of the treatment. These results indicate that the differentiation of the chemical signature of the worker caste is a dynamic process, depending only on time and not on colony membership, confirming that, for these termites, this signature has a lesser role in colony membership than caste membership, unlike the chemical signatures of other social insects. The temporal process of this cuticular change is also associated with a change in the alkene / methyl-branched alkane ratio.

Résumé. Synchronization endogène de la signature chimique des termites ouvriers du genre *Reticulitermes* (Isoptera : Rhinotermitidae). Les termites du genre *Reticulitermes* sont courants dans nos régions tempérées. Leurs sociétés sont composées de différentes castes, celle des ouvriers étant la plus abondante, les individus pouvant y évoluer en soldats ou reproducteurs secondaires. Chaque caste possède une mixture de composés cuticulaires (hydrocarbures) formant une signature chimique quantitative propre à chacune. Le but au départ de cette étude était de comparer la signature chromatographique d'ouvriers *Reticulitermes flavipes* nourris par de l'hormone juvénile pour suivre la modification de signature chimique lors de leur induction en soldat. L'expérience qui a duré plus d'un mois n'a pas permis la différentiation en soldat mais a permis de montrer que la signature chimique des ouvriers subissait une modification synchrone pour les 3 colonies analysées, quelque soit le traitement subi, l'environnement, et sans montrer de variations coloniales, à la différence des variations classiquement vues chez les autres insectes sociaux. Ce processus dynamique est également concomitant à la modification de proportions de différentes composés (méthylés versus insaturés) au cours des semaines.

Keywords: Cuticular hydrocarbons; *Reticulitermes flavipes*; cuticle dynamics, caste differentiation.

The chemical signature on the cuticle of social insects is a mosaic of hierarchical informations designed to maintain the integrity of their colonies (Howard & Blomquist 2005). Within an ant colony, nestmates recognize each other by wiping their antennae over the cuticle to create a uniform odor (Lenoir *et al.* 1999). The chemical signature is constituted mainly of hydrocarbons that form a complex mixture of tens of compounds in different proportions within each species.

The chemical signature of termites is an excellent marker for chemotaxonomy, particularly for subterranean termites (Rhinotermitidae) of the *Reticulitermes* genus (Bagnères & Wicker-Thomas 2010). In these termites, however, the signature seems to have a different hierarchical function from that in social hymenoptera, as chemical differences between colonies appear to be more subtle than differences between castes, which, in termites, have a real chemical identity (Bagnères *et al.* 1998; Landré 2008). The differences can be perceived by the antenna contact between termites followed by processing of the olfactive information within the central nervous system to generate the recognition behavior (Bagnères *et al.* 1998). The cuticular compounds may also play a role in caste regulation similar to primer pheromones (Clément & Bagnères 1998; LeConte & Hefetz 2008). Therefore, the chemical signature and its variations depend on a wide range of factors: endog-

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E-mail: bagneres@univ-tours.fr
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enous factors, such as endocrine or enzymatic factors and the regulation of transport paths, etc (Lengyel *et al.* 2007; Schal *et al.* 2003; Fan *et al.* 2004), as well as exogenous factors, such as climate and season (Bagnères *et al.* 1990), diet (Liang and Silverman 2000), the presence of predators, etc. The predominance of these factors varies between insects (VanderMeer & Morel 1998). However, this veritable mosaic of odors appears to be modified not only by the social or biotic environment but also by genetic control in social as well as solitary insects (Bonavita-Cougourdan *et al.* 1993; Vauchot *et al.* 1996; Dronnet *et al.* 2006; Thomas & Simmons, 2008; Etges *et al.* 2009).

Little is known about the process of regulating and secreting cuticular compounds (Bagnères & Blomquist 2010) and few studies have focused on how the chemical signature changes with time. In ants, for example, the cuticular hydrocarbon (CHC) profile of the colony changes significantly with time for the whole of a colonial group (Ichinose, 1991; Lenoir *et al.* 1991; Provost *et al.* 1993). However, there is a conflict between the existence, on the one hand, of a unique signature for each individual carrying a variety of information on its cuticle about its physiological condition, its role in the colony, its hierarchical position, its gender, etc., and, on the other, the existence of the specific signature carried by the colonial group, which forms the odor of a super-individual ("Gestalt model" defined by Crozier & Dix, 1979).

This study monitored the changes in the cuticular compounds during an experiment to differentiate worker termites into soldier termites induced by the juvenile hormone (JH-III). It has been shown that JH-III can induce differentiation into soldiers, partly by changes in the expression of the coding genes, for example for the muscular proteins of the mandibles (Hrdy & Krecek 1972; Park & Raina 2004; Scharf *et al.* 2003). Other studies have shown that this hormone regulates the synthesis and possibly the transport of CHCs in *Myrmicaria eumenoides* ants (Lengyel *et al.* 2007). In this study, the experiment to induce workers into soldiers by ingesting JH did not succeed in forming pre-soldiers in 35 days. The experiment carried out by Scharf *et al.* (2005) produced the first pre-soldiers of *R. flavipes* after day 14 but with a differentiation rate of only 3%. The experiment was repeated successfully later using a larger number of individuals and JH analogues.

Table 1. Numbering of samples and day numbers after t0 (see fig. 1).

Sampling Nb	Days after t0
0	t0
1	3d
2	6d
3	9d
4	13d
5	17d
6	21d
7	24d
8	29d
9	35d
F	35d

However, it has been possible, in several microcolonies formed by workers at similar stages, to follow the changes in their chemical signature with respect to that of control individuals, in similar and dissimilar environments. This study tested whether the CHC signature differed between colonies, changed with time or differed with respect to the controls.

Material and Methods

Biological material

The individuals used came from French colonies of *R. flavipes* (Kollar, 1837), previously known as *R. santonensis* (Feytaud, 1924) (Bagnères *et al.* 1990; Austin *et al.* 2005; Perdereau *et al.* a,b) in this area, collected on the Ile d'Oléron (Charente Maritime, dpt 17, France) between 2005 and 2007. At first they were kept in their original stump and were gradually transferred to large Plexiglas boxes (Boite Lab 3412®, LTD,

Table 2. Peak codes (a: *n*-alkane, m: monomethylalkane, d: dimethylakane, e: monoene, n: diene, x: unknown), full names and abbreviations.

Peak	Full name	Abbreviation
e1	9-Tricosene	9-C23:1
e2	Tricosene	x-C23:1
a3	*n*-Tricosane	*n*-C23
m4	11-Methyltricosane	11-MeC23
m7	4/2-Methyltricosane	4/2-MeC23
e8	9-Tetracosene	z,9-C24:1
m9	3-Methyltricosane	3-MeC23
a11	*n*-Tetracosane	*n*-C24
m12	11-Methyltetracosane	11/12-MeC24
m14	5-Methyltetracosane	5-MeC24
m16	4/2 Methyltetracosane	4/2-MeC24
e17	9-Pentacosene	z,9-C25:1
e18	Pentacosene + Pentacosadiene	x-C25:1
a19	*n*-Pentacosane	*n*-C25
x20	Unknown	x
m21	11/13-Methylpentacosane	11/13-MeC25
m24	5-Methylpentacosane	5-MeC25
n25	7,9-Pentacosadiene	7,9-C25:2
m26	4/2-Methylpentacosane	4/2-MeC25
m29	3-Methylpentacosane	3-MeC25
d30	5,17-Dimethylpentacosane	5,17-diMeC25
a31	n-Hexacosane	n-C26

France, 35 × 24 × 14) kept in the laboratory. A set of four 5 cm diameter Petri dishes, containing 7.5 g of Fontainebleau sand and 1.5 ml distilled water, was set up for each of the three different colonies (B, G and H) and 50 *R. flavipes* workers in terminal stages (6–7) were placed in each dish. A control set of two Petri dishes (T) with 50 individuals from colony B was also set up. The aim was that each termite would rapidly ingest 5 µg of JH-III (JH-III Sigma-Aldrich J2000-10MG) (Scharf *et al.* 2003). Twelve 2 × 2 cm pieces of Whatman No. 1442 070 filter paper, uniformly impregnated with a 20 µl solution of JH-III diluted in methanol at 2.5 g.l^{-1}, were prepared. Two pieces of paper impregnated only with methanol were prepared for the blank control dishes. After methanol had evaporated, the 14 pieces of filter paper were ready to be placed in the dishes.

One hundred individuals from each of the 3 colonies (B, G and H) were also put into Plexiglas boxes (Boite Lab 3412®, LTD, France, 12 × 9 × 5 cm) as environmental controls. All the Petri dishes and boxes were placed in an enclosure at 23 °C in the dark. The individuals were left in these dishes and boxes without food for 4 days before the start of the experiment.

After four days without food, the filter papers with or without JH were put onto the surface of the sand in the Petri dishes and moistened and then a piece of pinewood was placed in each of the 3 Plexiglas boxes. The individuals from each colony in each of the three Plexiglas boxes were sampled at the start of the experiment (t0) just before the paper impregnated with JH-III was placed in the Petri dishes and the wood was placed in the boxes. When the paper in a dish had been completely consumed, a piece of filter paper without JH-III was added to continue feeding. Every 3 to 5 days, five individuals sampled from the various experimental dishes were pooled, for each series. Three sets of five individuals were also taken from the Plexiglas boxes (F) on day 35 at the end of the experiment. Eleven sets of samples (see tab. 1) were taken and 42 chromatographic analyses were performed.

Chemical analysis

Extractions. The CHCs from each pool of five individuals were extracted in 200 µl of pentane in 2 successive 5 minute extractions, dried in nitrogen and then put into 14 µl of a pentane calibration solution (internal standard) containing Eicosane (*n*-C20) at 10^{-7} g.l^{-1}.

Chromatography and integration. 2 µl of extract were injected into the gas chromatograph (Agilent Technologies 6850 Network GC System). The temperature was programmed to start at 70 °C, increase by 30 °C/min up to 150 °C and then increase by 5 °C/min up to 315 °C, and the final temperature was maintained for 10 minutes at 315 °C. Agilent GC Chemstation (v. B.03.01.SR1.1) was used for data acquisition and processing.

The specific CHCs had already been identified by GC-MS (Bagnères *et al.* 1990). Twenty one peaks were quantified for *R. flavipes* with the numbering used by Clément *et al.* (2001) (tab. 2).

Data analysis

Peak areas were used to calculate the proportions of the CHCs to which FID correction factors were then applied (Bagnères *et al.* 1991a,b).

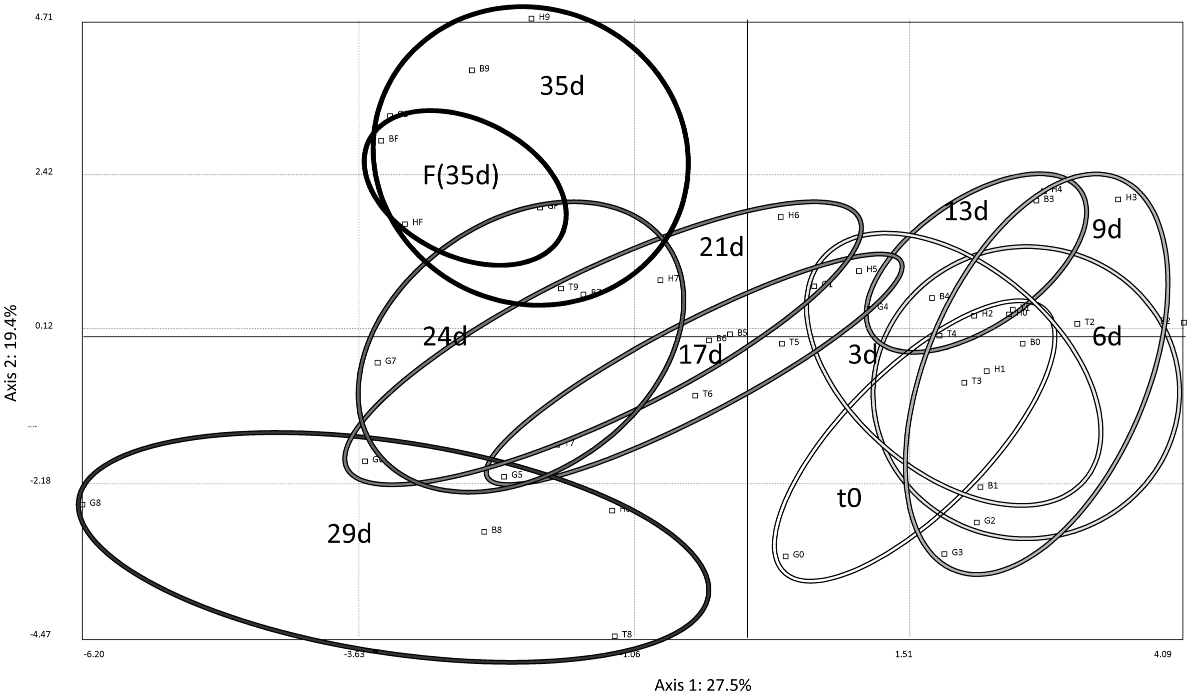

Figure 1A
PCA analysis showing the projection on the first 2 axes of the points for pools of individuals from colonies B, G and H, T (control) and F (from B, G and H control colonies). Circles show the clusters formed by time plots and have no statistical significance.

Multivariate Principal Component Analysis (PCA) was used to show the correlation between the *n* variables (hydrocarbons) and determine the main axes that best explained the dispersion of the cloud of points (pool of individuals). The variances (inertia) were attributed to these axes to account for their share of the total variance using Statgraphic Plus 4.0 and Uniwin Plus 3.01 software.

Results

The experiment did not lead to the differentiation of pre-soldiers (and therefore not into soldiers either) after 35 days in the 12 Petri dishes and 3 Plexiglas boxes. Looking at the plane formed by the first 2 PCA axes (fig. 1A), accounting for 27.5% and 19.4% of the variance respectively, the points for the extracts from the control Petri dishes (T) are not clearly distinct from the 3 points corresponding to the extracts of individuals from the 3 different colonies (B, G, and H) fed with JH. For each of the 10 different sampling dates, the 4 extracts could be grouped together in a statistically non-significant circle. Furthermore, there was a separation on axis 1 between the first 5 sampling dates (0 to 13 days) and the last 3 sampling dates (24 to 35 days), with intermediate sampling dates (17 and 21 days) being in the centre. This, therefore, showed a progression in the CHC profiles along axis 1 over time. A separation can also be seen between the 4 penultimate samples (T, B, G and H) taken at 29 days and the last samples at 35 days (the 4 samples (T, B, G and H) plus the 3 samples from the Plexiglas boxes (F), i.e. environmental controls) along axis 2. However, the points for each of the sampling dates were never grouped by colony in any of the factorial planes over the 10 sampling dates, although the points corresponding to the extractions of the four sets of different dishes (T, B, G and H) always seemed to be slightly separated and were, therefore, slightly discriminated (hence the statistical non-significance of the circles).

The last samples (F), taken on day 35 from each of the three Plexiglas boxes for each colony (B, G and H), were included within the circle (non significant) formed by the points for the last sample on day 35 from the Petri dishes (see fig. 1A). Only axis 3 (10%)(figure not shown) separated the colonies in the Plexiglas boxes at F slightly from the control (T) and colonies (B, H and G) in the Petri dishes at 9 (35d) but they were still systematically grouped on both axes regardless of the axes chosen.

The biplot (fig. 1B) shows that the first samples

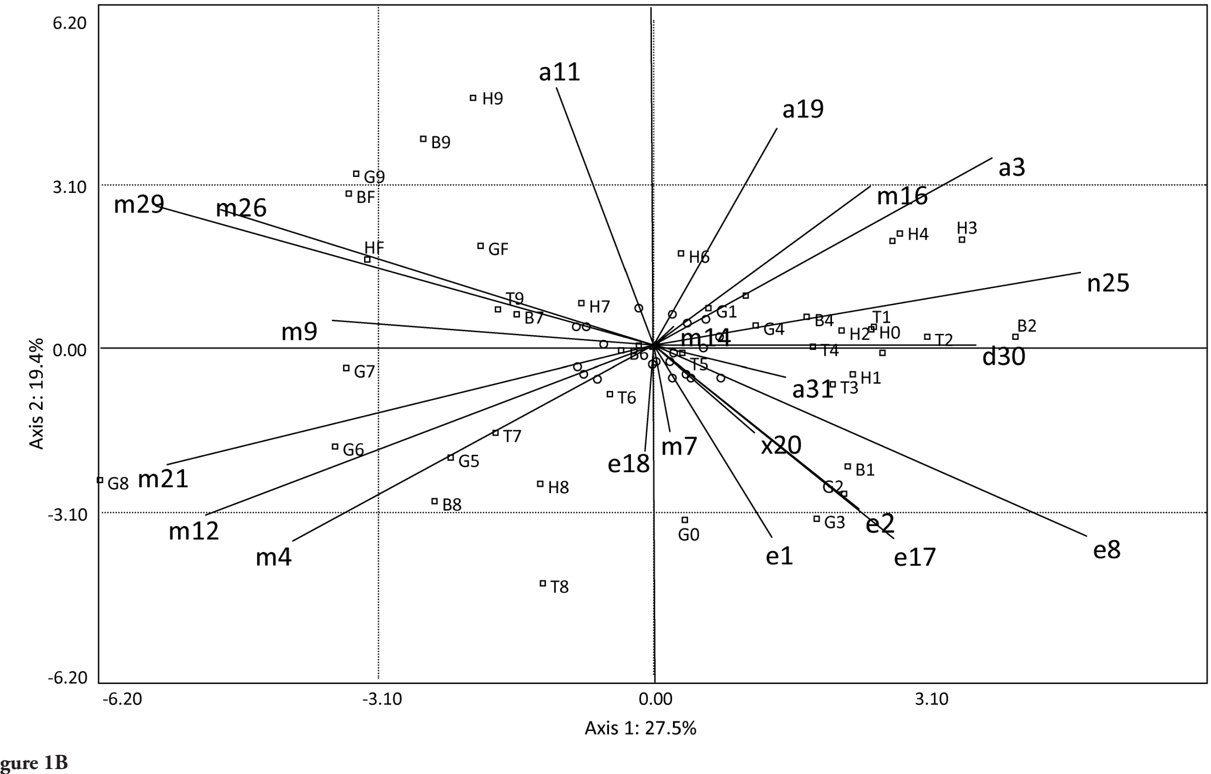

Figure 1B
PCA biplot showing the various vectors (hydrocarbons) involved in discriminating the time groups.

were discriminated by unsaturated compounds, monoenes (C23, C24 and C25: e1, e2, e8, e17, and to a lesser extent e18) and the conjugated diene (C25: n25), *n*-alkanes (C23, C25 and C26: a3, a19 and a31) and d30, m16 and x20. However, the last points were discriminated only by methylalkanes (C23, C24 and C25: m4, m9, m12, m21, m26 and m29). The points for the individuals at day 35 appear to be influenced mainly by a11, an *n*-alkane (C24).

Discussion

Results of this study show that the profile changed over time within the three experimental colonies and the control, independently of treatment. The results also show that the changes of the profile with time, in these experimental conditions, were greater than the difference between the colonies. The change in the chemical signature of termites confirms that the difference between colonies is not the major variation, as it is in other social insects such as ants or wasps, where the odor of the colony strongly predominates. As this variation occurred in the different species of Europe and native populations of *R. flavipes* in USA (Bagnères 1989; Bagnères *et al.* 1990), this could not be linked to the social characteristic of the French populations of *R. flavipes* with only open societies (Clément and Bagnères, 1998; Perdereau *et al.* 2010b).

These results appear to be independent of the development of the larval stages as each Petri dish contained workers at similar terminal stages. It may, therefore, have been the introduction of a new standardized environment (Fontainebleau sand, distilled water, darkness, 23 °C) that caused the start of a synchronized evolution of the profile for each different time, but this seems improbable. Moreover, the new environment, in particular the food factor, is certainly not determinant, given that the points of the individuals in the environmental controls from the Plexiglas boxes (F), fed with a piece of wood, are included in the circle enclosing the points for the individuals from the Petri dishes, fed with filter paper, taken at the same final time (blank controls and termites fed on JH-III at 35 days from the Petri dishes). At the end of the experiment, there were, therefore, no significant differences in CHCs owing to diet, as shown by Liang and Silverman (2000) for the Argentine ant, or by Etges *et al.* for the lack of influence of host plants and sexual maturity in drosophila CHCs (Etges *et al.* 2007, 2009). Furthermore, the workers in the Plexiglas boxes also evolved in a social environment different from the environments in the Petri dishes (more than a hundred in a Plexiglas box as against 50 in Petri dishes). Despite these differences in environment, their chemical signatures changed in the same way so that individuals at the end of the 35 day experiment were all close to each other on the plane defined by the first 2 axes (about 50% of variance). Finally, the various colonies were collected and sampled from their original stumps at different times and kept for a varying length of time before the experiment. It can, therefore, be suggested that there is an internal clock that changes the signatures synchronously over time, depending on the biosynthesis pathways and their regulation, or transport pathways and thus the changes are controlled by intrinsic factors rather than the temperature and humidity in this experiment, or other exogenous factors.

From the physical and chemical point of view, it is interesting to note the changes in the compounds themselves over time. For the first sampling dates, unsaturated compounds seemed to play a predominant role in discrimination. However, the last samples were discriminated only by monomethylalkanes. This could indicate that unsaturated compounds change faster over time, by undergoing a faster turnover, and that methylated compounds change more slowly. This is consistent with several studies of signatures with a high concentration of unsaturated compounds that are modified more rapidly (Bagnères *et al.*, 1996; Vauchot *et al.* 1997). It is also known that volatility and desiccation differ according to the nature of the compounds (Gibbs and Pomonis, 1995). The turnover of cuticular compounds has been studied in very few insects, mostly in solitary insects and in particular during the modification during the sexual maturity cycle. Kent *et al.* (2007) studied the diurnal cycles of *Drosophila melanogaster* males. Certain compounds seemed to have a greater variability. Mpuru *et al.* (2001) were able to trace, day by day, the formation of male and female signatures for *Musca domestica* linked directly to the hormonal cycle. In this case, the cycle (if it is really a cycle) of the whole of the signature seems much longer than a diurnal cycle and the way it changes does not appear to be linked to environmental conditions (light, temperature) or to physiological status (sex, state of maturity, hormones) but mainly to endogenous factors intrinsic to the physical and chemical changes in the cuticle.

Similarly, unlike the post-pharyngeal gland in ants, no gland has as yet been found in termites that could play an equivalent role in a turnover or homogenization process (Lenoir *et al.* 1999) whereas changes with time, caused by this gland, have been recorded among *Leptothorax* ants (Provost *et al.*, 1993) and in particular among *Camponotus* (Meskali *et al.*, 1996). However, we cannot eliminate the hypothesis that the absence of other castes in this experiment may have influenced this change in different ways.

Changes in profiles in termites, as well as in insects in general, are, therefore, not fully explained. In *R. flavipes,* it has been noted that the genetic distance did not account for the whole variation in hydrocarbons (Dronnet *et al.* 2006). Other factors could play a role in these changes, for example the action of the social environment is particularly visible and rapid in mixed artificial or natural colonies of ants or termites (Bagnères *et al.* 1991b; Vauchot *et al.* 1996; Bonavita-Cougourdan *et al.* 1996), with rapid cuticular adjustments. This could suggest a process of rapid feedback by transporting HCs, internalisation and metabolization, and a slower process via the hemolymph and lipophorins recycling the hydrocarbons. This process has not as yet been described but would explain, for example, the rapid elimination of exogenous or surplus compounds (Sevala *et al.* 2000, Vauchot *et al.* 1997).

Many stimuli could activate or inhibit the genes involved in the hydrocarbon biosynthesis pathways and/or in those of transport via lipophorins. The very little information available about this relates to drosophila (Wicker-Thomas & Chautemps 2010). Account must, however, be taken of the plasticity of the genomes *vis à vis* the biotic and abiotic environment in setting up the chemical signature as well as the direct action of the environment on the signatures, making them change over time in societies in the natural state.

There seems, therefore, to be a dynamic process leading to changes in the chemical signature of termites over time within a caste, a sort of 'caste Gestalt' similar to the model defined by Crozier & Dix (1979) explaining the formation of the uniform colonial odor in ants. This 'caste Gestalt' is read during incessant antenna exchanges between nestmates and may be subject, in termite workers, given that they are larval forms, to extensive monitoring to regulate and minimize the inward and outward flow of CHCs through their fine cuticle, thus forming a 'caste Gestalt template'. This would explain the relatively long cycle of about thirty days to give time for the whole of the group or nest to adjust and regulate between castes, while some members continue to molt. However, it is difficult to explain this "cycle" of around thirty days that forms a signature that is relatively different from the starting point. At 35 days, is there a return to the initial signature, a stationary state or the start of a new "cycle"? Longer studies are required. However, it can be concluded that the construction of the chemical signature, a complex mosaic of odors, is an extremely complex physiological and biochemical phenomenon, continuously changing during the insect's life, depending on the social environment, biotic factors, the physiological status, etc, about whose mechanisms very little is known.

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