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Nest structures display specific hydrocarbon profiles: insights into the chemical ecology of the invasive yellow-legged hornet *Vespa velutina nigrithorax*

Mélissa Haouzi[1], Jérémy Gévar[2], Alix Khalil [1], E. Darrouzet[1]*

[1]*IRBI, UMR CNRS 7261, University François Rabelais of Tours, Parc de Grandmont, 37200 Tours, France*

[2] *iEES Paris, INRA, 78026 Versailles, France*

*Corresponding author: eric.darrouzet@univ-tours.fr

ORCID:

MH: 0000-0002-8851-9223

ED: 0000-0002-7224-6937

Abstract

In insects, chemical communication is the most common form of communication, and cuticular hydrocarbons (CHCs) are employed in recognition processes. In social insects, CHCs also help define colony identity and thus contribute to social cohesion among nestmates. Individuals can deposit their chemical signatures on nest surfaces. This information serves as a reference for newly emerged individuals and allows them to obtain the odor specific to their colony. This study examined nest chemical profiles in an inbred invasive species: the yellow-legged hornet, *Vespa velutina nigrithorax.* We demonstrated that nest structures (i.e., envelopes, combs, and pillars) had specific hydrocarbon profiles, which were colony specific. There were similarities between the chemical profiles of the nests and the CHC signatures of hornets. The loss of genetic diversity previously documented in the yellow-legged hornet population in France does not appear to have constrained nest chemical diversity.

Keywords: cuticular hydrocarbons, chemical marking, Hymenoptera, invasive species

Introduction

The members of social insect colonies operate as a unit and are thus considered to form a higher-level organism (Wheeler 1911). The stability of social groups relies on group members being able to differentiate between those who belong to the group and those who do not (Hamilton 1987). This recognition system is based on chemical cues (Hölldobler 1995).

In insects, chemical communication can be carried out by pheromones, and substrates may be marked with volatile or non-volatile compounds. In social insects, nestmates can

communicate using cuticular hydrocarbons (CHCs). CHCs are non-volatile compounds and form a complex layer on the insect cuticle, providing essential protection against desiccation (Hadley 1984; Gibbs 1998). They also play a key role in kin recognition (Espelie and Hermann 1990), nestmate recognition (Hefetz 2007; D'Ettorre and Lenoir 2010), caste recognition (Howard et al. 1982; Smith and Taylor Jr 1990; Bagnères et al. 1998; Kaib et al. 2000), and the division of labor (Bonavita-Cougourdan et al. 1993; Scholl and Naug 2011; Rahman et al. 2016). The CHC signatures of insects are often complex and dynamic, with compound levels that vary qualitatively and quantitatively (Bonavita-Cougourdan et al. 1993; Lenoir et al. 1999, 2001). Indeed, insects have personalized chemical signatures that are shaped by endogenous factors (Fan et al. 2004; Lengyel et al. 2007; Darrouzet et al. 2014), exogenous factors (Bagnères et al. 1990), and parasitism (Bagnères et al. 1996; Darrouzet et al. 2010; Lebreton et al. 2010). An individual's chemical signature serves as a type of highly specific "visa," conveying the individual's identity to conspecifics (Fresneau and Errard 1994).

Some invasive social insects are tolerant of small differences in CHC signatures, a trait that enhances their invasive success (Suarez et al. 1999; Tsutsui et al. 2001; Pedersen et al. 2006; Brandt et al. 2009). In such species, colonies differ less in their chemical profiles and thus compete less for resources. One example of this phenomenon is the Argentine ant, *Linepithema humile*, which has successfully colonized several continents (Holway and Case 1999; Suarez et al. 2000). In this context, attention has turned to a species that recently invaded Europe: the yellow-legged hornet, *Vespa velutina nigrithorax*. This hornet is native to China and, more specifically, comes from two provinces near Shanghai (Arca 2012; Arca et al. 2015). It was accidentally introduced into southwestern France around 2004 (Haxaire et al. 2006) and then spread rapidly throughout Europe (Robinet et al. 2018). It is now found in Spain, Portugal, Italy, Germany, and, more recently, in Belgium, the Netherlands, Switzerland, and the United Kingdom (Bruneau 2011; López et al. 2011; Grosso-silva and Maia 2012; Demichelis et al. 2014; Monceau and Thiéry 2017; Robinet et al. 2017, 2018). To feed its larvae, it consumes large quantities of insects (Villemant et al. 2011) and, more particularly, of honey bees (Darrouzet and Gévar 2014; Darrouzet 2019). Bee colonies suffer as a result, and their foraging activity decreases, thus impacting beekeeping (Requier et al. 2018, 2019; Leza et al. 2019). Not only is the yellow-legged hornet a threat to biodiversity, but it is also responsible for drastic economic losses as well as public health concerns (Beggs et al. 2011; Darrouzet 2019; Laborde-Castérot et al. 2020).

Despite the lack of genetic diversity in the European yellow-legged hornet population (Arca et al. 2015; Darrouzet et al. 2015), hornet chemical signatures, composed of CHCs, were

found to vary tremendously based on caste, sex, and colony (Gévar et al. 2017). This result was surprising because such heterogeneity has not been observed in other invasive social insects, such as ants [*Linepithema humile* (Tsutsui et al. 2000; Brandt et al. 2009); *Wasmannia auropunctata* (Errard et al. 2005)] or termites [*Reticulitermes flavipes* (Perdereau et al. 2010)]. Previous studies have suggested that this lower level of chemical variability may arise from the reduced genetic diversity that can result from genetic bottlenecks (Tsutsui et al. 2001) or selection against less-common recognition alleles (Giraud et al. 2002). Gévar et al. (2017) suggested that the heterogeneity of CHC profiles in *Vespa velutina nigrithorax* may be affected by environmental and/or epigenetic factors. For example, in termites, changes in temperature or diet can erase or modify colony odor in *Coptotermes formosanus* (Shelton and Grace 1997; Florane et al. 2004). In *Reticulitermes flavipes*, seasonal variation can modify chemical signatures (Bagnères et al. 1990); in *Cryptotermes brevis*, profiles shift depending on specific combinations of temperature and relative humidity (Woodrow et al. 2000). The composition of the intestinal bacteria community can also affect nestmate recognition in *Reticulitermes speratus* (Matsuura 2001, 2003).

In eusocial insects, nest odors are present and attractive to colony members (Kukuk et al. 1977; Gamboa et al. 1986; Ferguson et al. 1987). Indeed, colony odor helps maintain colony unity in ants and termites (Wilson 1971). Insects may use visual cues for nest recognition, but olfactory cues have also been observed in *Apis mellifera* (Butler et al. 1969, 1970; Free 1987) and *Lasioglossum zephyrum* (Kukuk et al. 1977). Cuticular hydrocarbons may be deposited on nest structures, like the walls and soil surfaces of ant nests (Bos et al. 2011), the waxy combs of beehives (Couvillon et al. 2007), and the paper nests of social wasps (Espelie and Hermann 1990; Elia et al. 2017). For example, the ant *Lasius niger* passively deposits CHCs on the inner walls of its nest via its tarsi (Devigne and Detrain 2006; Lenoir et al. 2009). *Camponotus aethiops* indirectly transfers CHCs to its nest, creating a uniform colony odor (Bos et al. 2011). In the social wasp *Polistes metricus*, the CHCs of adult wasps may be similar to those of the nest (Espelie et al. 1990). The chemical compounds laid down within the nest allow newly emerged individuals to recognize and obtain their colony's signature (Singer and Espelie 1992). In the social wasp *Polistes biglumis*, there is temporal variation in nest chemical profiles, which can also shift when colonies are invaded by the social parasite *Polistes atrimandibularis* (Elia et al. 2017).

This study aimed to investigate (1) whether nests built by *Vespa velutina nigrithorax* displayed hydrocarbon-based chemical profiles, (2) whether the three main nest structures (envelopes, combs, and pillars) had similar or different chemical profiles, and (3) whether nests

differed in their chemical profiles. We hypothesized that, since hornet workers display colony-specific CHC signatures (Gévar et al. 2017), the latter should be reflected in the chemical profiles of nests, since hydrocarbons should be deposited onto nest materials. This process could influence colony signatures across generations (Singer and Espelie 1992), overcoming the effects of inbreeding within the invasive population (Darrouzet et al. 2015).

Materials and Methods

Vespidae nests are built with plant fibers mixed with water and saliva (Edwards 1980). Yellow-legged hornet nests are located in different habitats but frequently occur in tree tops; they can generally be distinguished from the nests of other social Vespidae in France by the presence of a lateral entrance (Rome et al. 2009; Darrouzet 2013, 2019). Nests are composed of a stack of several combs that are connected by pillars and covered by a protective envelope (Darrouzet 2019). The combs are composed of alveolar cells that open downward; these cells harbor larvae and nymphs at different developmental stages. The top of the nest does not contain cells; instead, it consists of a sponge-like structure that likely protects the lower part of the nest (where the hornets are found) against harsh environmental conditions (e.g., rain). Nest construction follows a cyclical pattern and varies depending on the colony's degree of development. After overwintering, the foundress will begin nest building in early spring (March–April): she will first affix the pedicel and then build an initial comb of a dozen alveolar cells that are surrounded by a protective envelope. Around May, workers will emerge. They will enlarge and strengthen the nest along the vertical axis by adding new combs and along the horizontal axis by adding new cells to already established combs (Rome et al. 2015; Darrouzet 2019).

Chemical analysis of different nest structures

Nest collection

A total of 10 *Vespa velutina nigrithorax* nests (Table S1) were collected in France's Centre-Val-de-Loire region between 2015 and 2017. Each nest was processed immediately and stored at -20°C. Chemical extractions were carried out in 2018.

Hydrocarbon extractions

Hydrocarbons were extracted from three distinct nest structures: the envelopes, the combs, and the pillars. For each structure, three replicates were performed per nest (n = 10 nests). To extract the hydrocarbons, 150 mg of structural material was placed in a 20-ml glass vial and crushed

down to small particles. Then, 5 ml of heptane was added, and the whole mixture was vortexed for 1 minute at 300 rpm. It was left to rest for 24 hours, allowing the particles to settle. Subsequently, 1.5 ml of the supernatant was transferred into 2-ml glass vials and fully evaporated under nitrogen flux. Then, 150 µl of heptane was added to each vial, and the mixture was vortexed for 30 seconds at 300 rpm. Afterward, 100-µl aliquots of these extracts were transferred into vials. Before the chemical analyses were performed, 3 µl of C20 (n-eicosane 10^{-3} g/ml) was added as an internal standard.

Detailed comb analyses

Nest collection and sampling

To carry out a more detailed chemical analysis of the nests, three end-of-season nests (i.e., large nests; Table S2) were collected in the Centre-Val-de-Loire, Brittany, and Pays-de-la-Loire regions from February to March 2018. The combs' hydrocarbon profiles were analyzed by taking samples from different parts of the nest: from the top comb to the bottom comb and from the center, middle, and periphery of the individual combs. In each case, three replicates were performed (n = 3 nests).

Hydrocarbon extraction

To extract the hydrocarbons, 150 mg of structural material was collected. Then, 5 ml of heptane was added. The whole mixture was vortexed for 1 minute at 300 rpm and left to rest for 24 hours. Subsequently, 100 µl of the supernatant was transferred to 2-ml glass vials containing an insert. Before the chemical analyses were performed, 3 µl of the internal standard (C20 n-eicosane at 10^{-3} g/ml) was added to samples.

Chemical analyses

Samples were analyzed using gas chromatography (GC), employing an Agilent Technologies 7000C GC/MS Triple Quad with a 7890 BGC System equipped with a capillary column (HP-5 Agilent Technology, Santa Clara, USA; 30 m × 0.32 mm × 0.25 µm); helium was the carrier gas (1.7 ml/min). The following temperature program was used: an initial temperature ramp from 50 °C to 200 °C at 8 °C/min, a second temperature ramp from 200 °C to 315 °C at 5 °C/min, and a 5-min final hold at 315 °C. The surface areas of the 27 major peaks were determined using ChemStation software (Agilent Technologies). The hydrocarbon profiles of the nests were compared to the species' chemical signature. The identities of the chemical compounds present on the nest structures were determined via comparison with the *Vespa*

velutina nigrithorax CHCs analyzed using the same GC system (Gévar et al. 2017). Compound identities were confirmed using GC-MS analyses performed with an Agilent Technologies 7000C GC/MS Triple Quad with a 7890B GC System equipped with the same column as above; we also used the same temperature program as above.

Calculations and statistical analyses

Principal component analysis (PCA) was carried out using R software (v. 3.4.3) and the packages FactoMineR (Lê et al. 2008) and RcmdrPluginFactoMineR (Fox 2005), which are included in the software. Differences in the hydrocarbon profiles of the three nest structures (envelopes, combs, and pillars) among and within nests were quantified using the Nei distance (Nowbahari et al. 1990; Dronnet et al. 2006). A dissimilarity matrix, which included all possible pairwise comparisons (within structures, among structures within nests, and among nests for a given structure), was constructed using the mean relative areas of the hydrocarbon peaks. The matrix was then analyzed using a Kruskal-Wallis test implemented in RStudio (v. 1.0.143; ©2009–2016 RStudio, Inc). For each structure, hydrocarbon quantity was estimated using the following formula: Qi = (QC20 X xi)/(xC20i) (Gévar et al. 2017). The quantity of each hydrocarbon was then divided by the mass of the nest sample to obtain the mass-specific quantity of each compound. Then, Kruskal-Wallis nonparametric tests were performed using RStudio software.

Results

Hydrocarbon analyses

The nest structures bore the same hydrocarbon compounds as the cuticle of *Vespa velutina nigrithorax* (Gévar et al. 2017) (Table 1). There were 8 alkanes, 10 methyl alkanes, 3 dimethyl alkanes, 1 di/trimethyl alkane, and 5 alkenes among the 27 major peaks analyzed.

Differences in nest structure hydrocarbons

The three nest structures (envelopes, combs, and pillars) had distinct hydrocarbon profiles (Fig. 1) in which certain compounds predominated. In particular, there were two alkanes and three methyl alkanes specifically found in the envelopes and combs; one alkane and one methyl alkane specifically found in the combs and pillars; and one alkane, five methyl alkanes, two dimethyl alkanes, and two alkenes specifically found in the envelopes and pillars (Table 2).

Chemical distances for nest structures

Chemical distances were compared within and among nest structures (Fig. S1). Chemical distances within structures (i.e., among samples from a given structure, regardless of nest identity) were significantly smaller than chemical distances among structures (i.e., among nest structures within the same nest, regardless of nest identity) (Kruskal-Wallis; p-value < 0.001). Similarly, for each structure, chemical distances among nests were significantly greater than chemical distances within structures (Kruskal-Wallis; p-value < 0.001).

Quantitative analyses of the nest structures

The envelopes had significantly lower quantities of total hydrocarbons than did the other two structures (Kruskal-Wallis; p-value < 0.001). The combs had significantly higher quantities of total hydrocarbons than did the pillars (Kruskal-Wallis; p-value < 0.05) (Fig. 2).

The structures all differed from each other in specific hydrocarbon quantities (Kruskal-Wallis; p-value < 0.001 for most comparisons, with p-value < 0.05 for the comparisons of alkanes and branched alkanes between the pillars and combs). For each compound, the envelopes displayed the lowest quantities, and the combs displayed the highest quantities (Fig. 3).

Qualitative analyses of the combs

Within combs, we found no significant differences among center samples or between center and middle samples (regardless of comb or nest) (Kruskal-Wallis; p-value > 0.05; Fig. S2). The chemical distances between center and periphery samples were significantly higher than the chemical distances among center samples (Kruskal-Wallis; p-value < 0.001; Fig. S2). Finally, the chemical distances among middle samples and among periphery samples were significantly different as were the chemical distances between middle and periphery samples (Kruskal-Wallis; p-value < 0.001; Fig. S2).

The results for the chemical distances among combs (Fig. S3) revealed that only comb 1 (the oldest comb, found at the top of the nest) was significantly different from all the others (Kruskal-Wallis; p-value < 0.05), with the exception of comb 7 (Kruskal-Wallis; p-value > 0.05). The other combs were not significantly different from each other (Kruskal-Wallis; p-value > 0.05).

Quantitative analyses of the combs

Within combs, hydrocarbon quantities decreased significantly from the center to the periphery (Kruskal-Wallis; p-value < 0.001 for the center/periphery and middle/periphery comparisons and p-value < 0.05 for center/middle comparison) (Fig. 4).

Across combs, hydrocarbon quantities differed significantly between combs 1, 2, 3, and 4 and combs 6 and 7 (Kruskal-Wallis; p-value < 0.05); comb 5 was only significantly different from comb 7 (Kruskal-Wallis; p-value < 0.05) (Fig. 5).

Discussion

Many studies have shown that nest recognition in social insects is based on chemical cues. Such is the case in bumble bees (Saleh et al. 2007), in honey bees and wasps (Butler et al. 1969), and in ants (Depickère et al. 2004). In this study, hydrocarbons previously found on the hornet's cuticle (Gévar et al. 2017) were discovered to occur on different parts of the hornet's nest. This finding suggests *Vespa velutina nigrithorax* deposits CHCs on nest structures. However, we do not know if deposition is active or passive. Indeed, no data on *Vespa velutina* behavior within the nest are currently available. Consequently, we do not know if the hornet interacts in a particular way with the nest substrate. Some species are known to directly interact with nest materials and/or with nestmates to obtain/deposit chemical markings (Akino 2008). For example, some European *Polistes* species apply chemical markings via rubbing (Turillazzi and Ugolini 1979). Conversely, in *Lasius niger*, ants passively mark the nest with their tarsi as they walk across the substrate (Depickère et al. 2004; Devigne and Detrain 2006; Lenoir et al. 2009). Further behavioral studies are thus needed to clarify marking mechanisms in *V. velutina*.

Chemical signatures are colony and caste specific in *V. velutina* (Gévar et al. 2017). In our study, we observed pronounced qualitative and quantitative chemical differences among nest structures. This finding may indicate that individuals differing in caste or reproductive status (fertile vs. non-fertile females) occur in separate parts of the nest. For example, chemical signatures can vary among castes: *V. velutina* queens bear four specific alkenes and have higher levels of CHCs than do other castes (Gévar et al. 2017). However, the queen-specific compounds were not found on the nest structures. The chemical signatures of nest structures are likely shaped by the chemical deposits of colony members, whose CHC signatures vary in their absolute and relative composition. This variation is functional and is likely influenced by a variety of recognition-oriented behaviors and discrimination among individuals (Hölldobler and Carlin 1987). For example, monomethyl alkanes often serve as signals (Nelson 1993). Hydrocarbons could therefore function in recognition and communication within nests. Indeed,

the nest's interior is opaque, creating dark conditions. Individuals thus need a mechanism other than vision to locate each other within the nest. Labeling nest structures so that they have different chemical profiles could allow insects to orient themselves and communicate with each other. The fact that combs and envelopes had the highest and lowest quantities of hydrocarbons, respectively, might support this hypothesis. However, these differences could also arise from the higher contact frequencies among individuals and between individuals and comb surfaces inside the nest. Furthermore, there could be smaller quantities of hydrocarbons on the envelopes because they are exposed to exterior conditions (e.g., rainfall could wash them away). More experiments are necessary to explore these issues. Moreover, nest structures were also qualitatively different from each other. This finding is interesting because branched alkanes and alkenes help define the chemical identity of colonies and are involved in communication among individuals (Howard and Blomquist 2005). Linear alkanes may play more of a role in physically protecting individuals against desiccation (Hadley 1984; Singer 1998). Consequently, their high levels on the nest's surface could provide protection against wet and humid conditions. Indeed, because the hornet's nest is composed of "paper mache", it is likely that chemical protection would be required. When n-alkanes and monomethyl alkanes are combined, they produce an impermeable layer whose solid-liquid phase transition extends over a wide temperature range (Gibbs 1995), allowing the nest to benefit from seasonal waterproofing. Finally, the chemical heterogeneity of nest structures could be explained by temporal shifts. In *Bombus terrestris*, cuticular lipids convey colony identity and change over time (Rottler-Hoermann et al. 2016). Further research is needed to clarify the chemical dynamics of nests.

We also found that there was spatial heterogeneity in the combs' chemical profiles. Hydrocarbon quantity decreased from the center to the periphery of the combs and from the top combs to the bottom combs. It appeared that the oldest parts of the nest (i.e., the center of the comb and the top comb) had greater quantities of hydrocarbons. This observation could be explained by structure age, these structures had spent more time in contact with adult hornets. There were qualitative differences along the horizontal axis of the individual combs, signaling differences associated with time of construction. In contrast, there were no differences along the vertical axis of the combs. These results suggest that marking patterns within nests may be somewhat complex. Further research is needed to clarify hydrocarbon deposition mechanisms and dynamics within nests and across time.

Finally, the chemical diversity among nests and among structures highlights the existence of a colony signature. Indeed, chemical diversity among nests was high. As described by Gévar et al. (2017), individual *V. velutina* hornets vary in their chemical signatures, and we

observed the same variability in the species' nests. Consequently, the chemical heterogeneity observed among individuals could explain the heterogeneity seen in the nests themselves. This result was somewhat surprising, however, because hornet populations in France are highly related (Darrouzet et al. 2015) as the result of a genetic bottleneck (Arca et al. 2015). Previous studies of invasive species all found that chemical variability was reduced in introduced populations. Indeed, this is the case in the ants *Linepithema humile* (Brandt et al. 2009) and *Wasmannia auropunctata* (Errard et al. 2005) as well as in the termite *Reticulitermes flavipes* (Perdereau et al. 2010). This phenomenon could be related to the limited number of introductions that have taken place. In the case of *V. velutina* in France, the population is inbred; the heterogeneity in hydrocarbon profiles within nests could result from environmental and/or epigenetic factors (Gévar et al. 2017). Additional research is clearly necessary to assess the roles played by different factors in shaping nest chemical profiles.

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Legends

Table 1. Identities and relative proportions of the hydrocarbons found on the three nest structures: envelopes, combs, and pillars.

Table 2. Differences in hydrocarbon quantities among nest structures (Kruskal-Wallis chi-squared test). Levels of 5 compounds differed between the envelopes and combs; levels of 10 compounds differed between the envelopes and pillars; and levels of 2 compounds differed between the combs and pillars.

Figure 1. Results of the principal component analysis of the proportion of hydrocarbons found on the three nest structures; the first two axes are shown. Three comparisons were performed: envelopes vs. combs, envelopes vs. pillars, and combs vs. pillars.

Figure 2. Total hydrocarbon quantities found on the envelopes, combs, and pillars (mg of hydrocarbons/gram of nest material).

Figure 3. Quantities of alkanes, branched alkanes, and alkenes found on the envelopes, combs, and pillars (mg of hydrocarbons/gram of nest material).

Figure 4. Total hydrocarbon quantities (mg of hydrocarbons/gram of nest material) at the comb's center, middle, and periphery.

Figure 5. Total hydrocarbon quantities (mg of hydrocarbons/gram of nest material) from the top comb to the bottom comb within nests.

Supplementary materials

Table S1. Collection locations and dates for the nests used in the chemical analyses of nest structures.

606 **Table S2.** Collection locations and dates for the nests used in the detailed chemical analyses of
607 the combs.
608 **Figure S1.** Chemical distances between the hydrocarbon profiles of the three nest structures:
609 envelopes, combs, and pillars. The among-nest chemical distances represent the chemical
610 diversity of the same structure across different nests; the among-structure chemical distances
611 represent the chemical diversity among nest structures within the same nest; and the within-
612 structure chemical distances represent the chemical diversity within a specific structure for a
613 given nest.
614 **Figure S2.** Chemical distances between hydrocarbon profiles for the comb center, middle, and
615 periphery. The within-zone chemical distances represent the distances among samples taken
616 from within the same comb zone; the between-zone chemical distances represent the distances
617 among samples taken from different comb zones.
618 **Figure S3.** Chemical distances between hydrocarbon profiles within combs from nest top to
619 bottom.
620

Table S1

Nest number	Latitude	Longitude	Collection date
1	47.401366	0.66336	07/13/2017
2	47.388264	0.827383	08/14/2017
3	47.413326	0.984407	08/17/2017
4	47.498614	0.57948	08/24/2017
5	47.543367	0.744155	10/06/2017
6	47.388264	0.827383	12/01/2017
7	47.388309	0.658258	07/29/2016
8	47.351861	0.66131	08/05/2016
9	47.394144	0.68484	08/29/2016
10	47.388264	0.827383	07/07/2015

Table S2

Nest Number	Latitude	Longitude	Height (cm)	Width (cm)	Comb number	Collection date
1	48.066152	-2.967055	73	41	10	20.02.2018
2	47.3404240	-1.526116	53	47	7	14.02.2018
3	47.3898439	0.7224790	44	40	7	20.03.2018

Table 1

RT (min)	Compound Identity	ECL	Envelopes		Combs		Pillars	
			%	+-SD	%	+-SD	%	+-SD
23.8	n-C20	20	X	X	X	X	X	X
28.8	n-C23	23	3.57	0.56	1.92	0.11	2.62	0.13
31.5	C25 :1	24.72	0.68	0.11	0.76	0.10	0.66	0.04
31.9	n-C25	25	16.49	0.53	15.72	0.84	17.23	0.32
32.4	13-+11-MeC25	25.37	1.86	0.19	1.51	0.09	2.14	0.13
32.7	5-MeC25	25.47	0.36	0.11	0.33	0.03	0.35	0.04
33.1	3-MeC25	25.70	3.88	0.22	3.10	0.16	3.28	0.19
33.5	n-C26	26	1.72	0.05	1.59	0.13	1.60	0.09
34.1	12-+10-MeC26	26.31	0.55	0.14	0.45	0.04	0.50	0.03
34.7	C27 :1	26.75	4.30	0.35	5.25	0.46	4.94	0.22
34.8	C27 :1	26.83	1.14	0.14	1.07	0.10	0.92	0.06

35.1	n-C27	27	23.71	1.28	32.01	2.58	29.63	0.96
35.5	13-+11-+9MeC27	27.32	4.66	0.56	4.91	0.87	5.82	0.71
35.6	7,MeC27	27.37	0.90	0.23	0.80	0.06	0.87	0.08
35.9	11,15-diMeC27	27.64	4.04	0.52	2.77	0.27	2.65	0.16
36.1	3-MeC27	27.81	8.41	0.31	6.38	0.74	7.48	0.22
36.2	5,15-diMeC27	27.89	1.13	0.49	1.68	0.64	0.95	0.11
36.4	n-C28	28	3.00	150	0.79	0.07	0.87	0.08
36.7	diMe or triMeC27	28.13	1.05	0.23	1.02	0.16	0.97	0.12
36.9	12-MeC28	28.35	2.33	0.47	0.62	0.18	0.48	0.04
37.6	C29 :1	28.78	2.32	0.15	3.24	0.36	3.59	0.14
37.7	C29 :1	28.84	1.13	0.17	2.08	0.41	1.29	0.08
37.9	n-C29	29	5.39	0.39	5.80	0.66	5.43	0.25
38.4	13-+11-+9-MeC29	29.37	1.81	0.18	2.21	0.23	2.61	0.07
38.8	11,15-+11,17-diMeC29	29.68	1.80	0.26	1.37	0.32	1.06	0.15
39.7	4-MeC30	30.44	0.64	0.22	0.20	0.05	0.32	0.14
40.7	n-C31	31	3.32	1.05	2.39	0.37	1.69	0.13

628

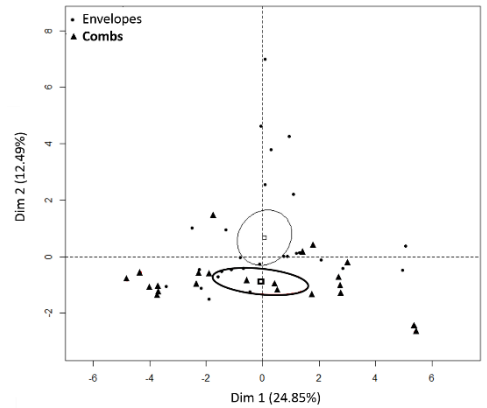
629 **Table 2**

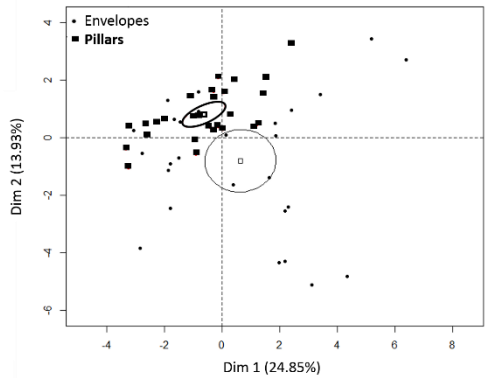
Compound	Structure comparison		
	Envelopes/Combs	**Envelopes/Pillars**	**Combs/Pillars**
n-C23	NS	NS	$p < 0.01$
C25 :1	NS	NS	NS
n-C25	NS	NS	NS
13-+11-MeC25	NS	$p = 0.01$	$p < 0.01$
5-MeC25	NS	NS	NS
3-MeC25	$p = 0.01$	$p < 0.01$	NS
n-C26	NS	NS	NS
12-+10-MeC26	NS	NS	NS
C27 :1	NS	$p = 0.01$	NS
C27 :1	NS	NS	NS
n-C27	$p < 0.05$	$p < 0.01$	NS
13-+11-+9MeC27	NS	NS	NS
7,MeC27	NS	NS	NS

11,15-diMeC27	NS	$p < 0.05$	NS
3-MeC27	$p < 0.05$	$p = 0.01$	NS
5,15-diMeC27	NS	NS	NS
n-C28	$p < 0.01$	NS	NS
diMe or triMeC27	NS	NS	NS
12-MeC28	$p < 0.05$	$p < 0.01$	NS
C29 :1	NS	$p < 0.01$	NS
C29 :1	NS	NS	NS
n-C29	NS	NS	NS
13-+11-+9-MeC29	NS	$p < 0.01$	NS
11,15-+11,17-diMeC29	NS	$p = 0.05$	NS
4-MeC30	NS	NS	NS
n-C31	NS	NS	NS

630

Figure 1


Envelopes
Combs
Dim 2 (12.49%)
Dim 1 (24.85%)


Envelopes
Pillars
Dim 2 (13.93%)
Dim 1 (24.85%)

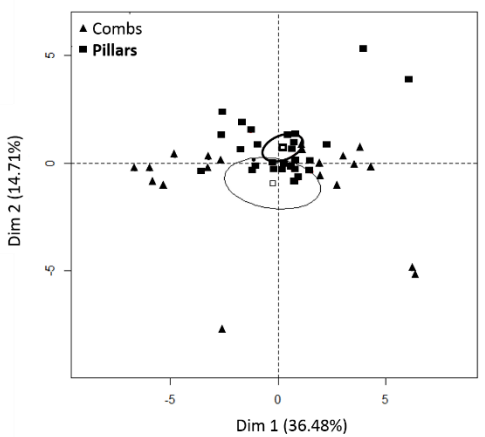
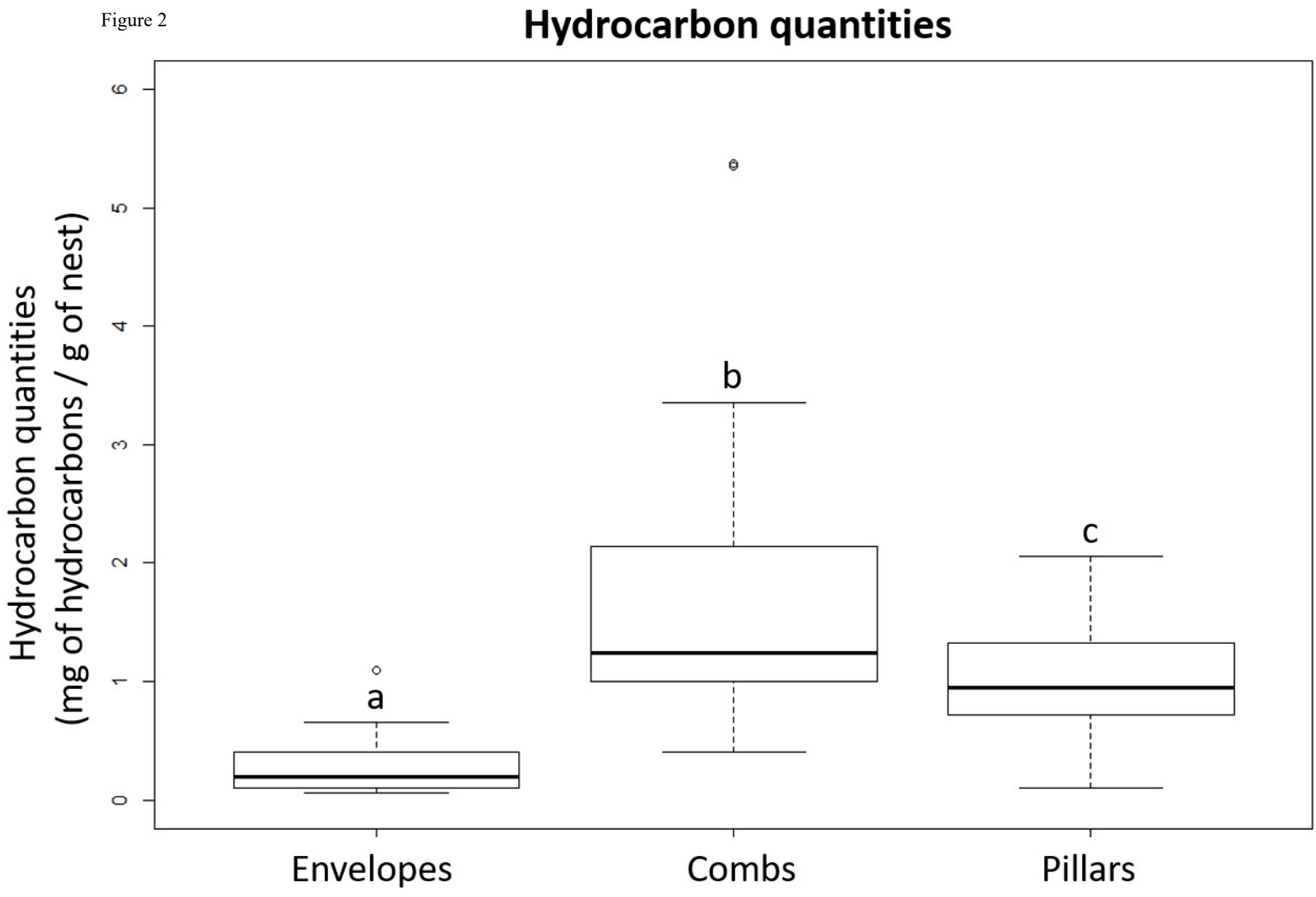
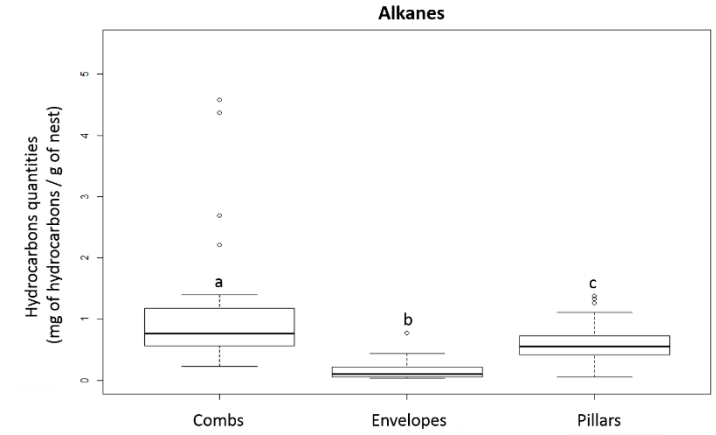
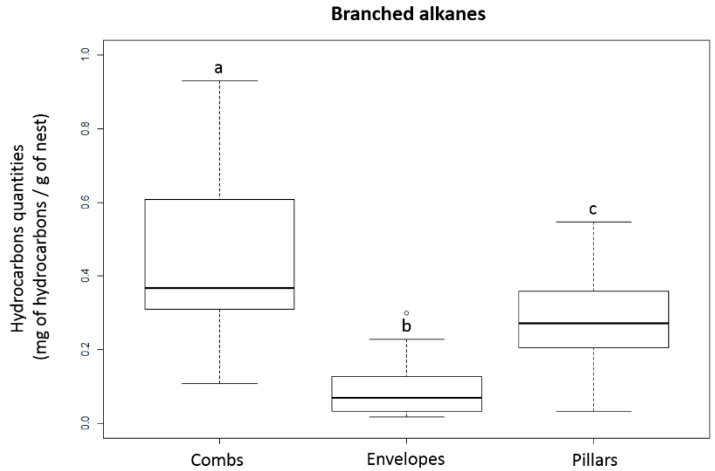

Combs
Pillars
Dim 2 (14.71%)
Dim 1 (36.48%)

Figure 2


Hydrocarbon quantities
Hydrocarbon quantities
(mg of hydrocarbons / g of nest)
0
1
2
3
4
5
6
a
b
c
Envelopes
Combs
Pillars


Alkanes
Hydrocarbons quantities
(mg of hydrocarbons / g of nest)
0
1
2
3
4
5
a
b
c
Combs
Envelopes
Pillars


Branched alkanes
Hydrocarbons quantities
(mg of hydrocarbons / g of nest)
0.0
0.2
0.4
0.6
0.8
1.0
a
b
c
Combs
Envelopes
Pillars

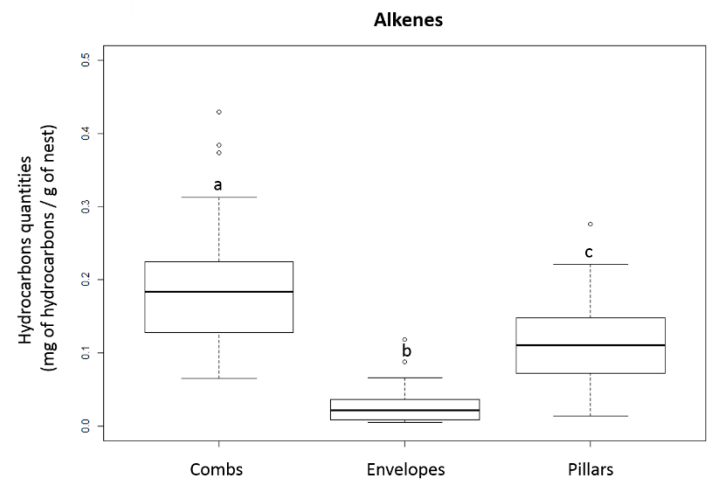

Alkenes
Hydrocarbons quantities
(mg of hydrocarbons / g of nest)
0.0
0.1
0.2
0.3
0.4
0.5
a
b
c
Combs
Envelopes
Pillars

Figure 3

Figure 4

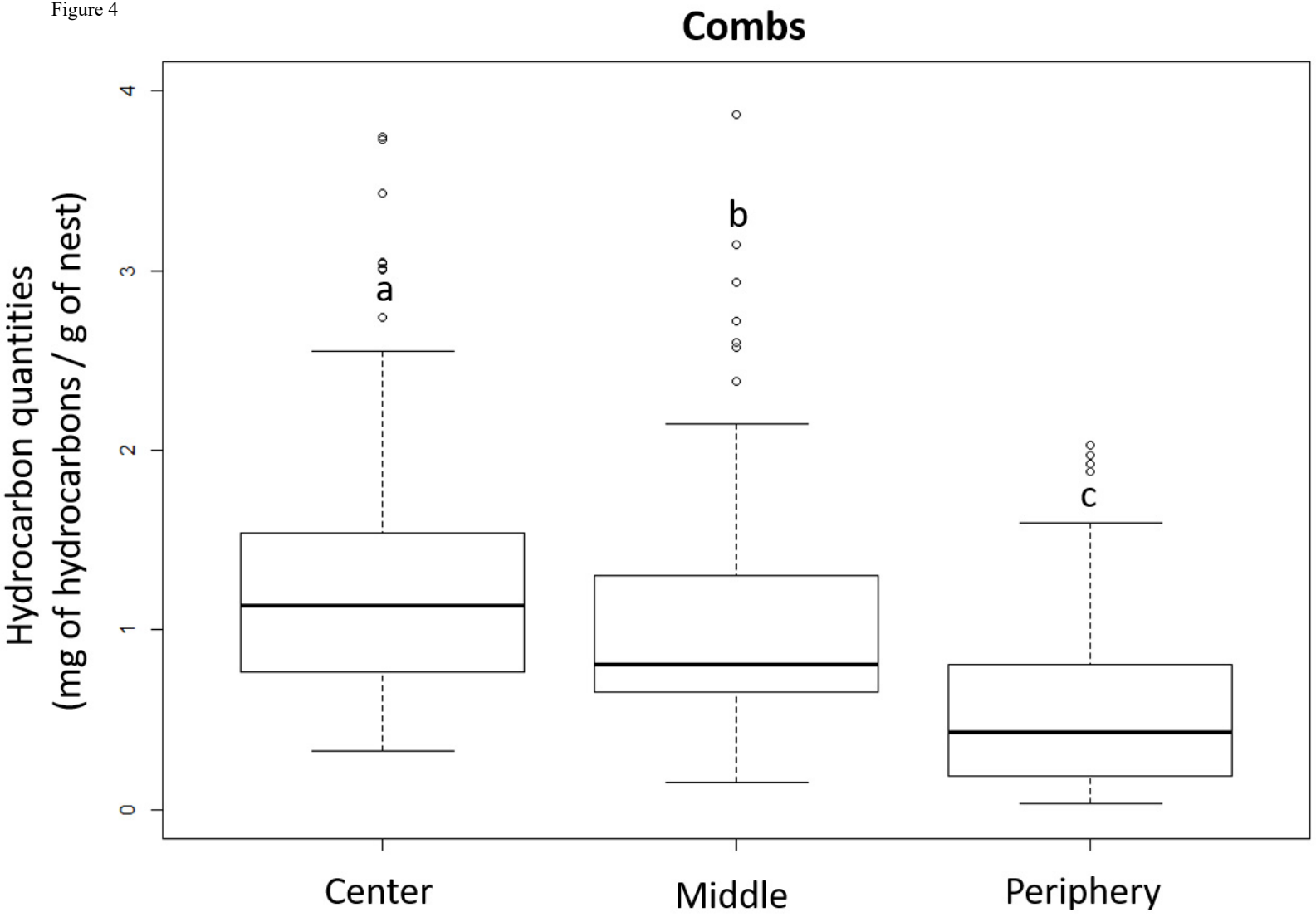
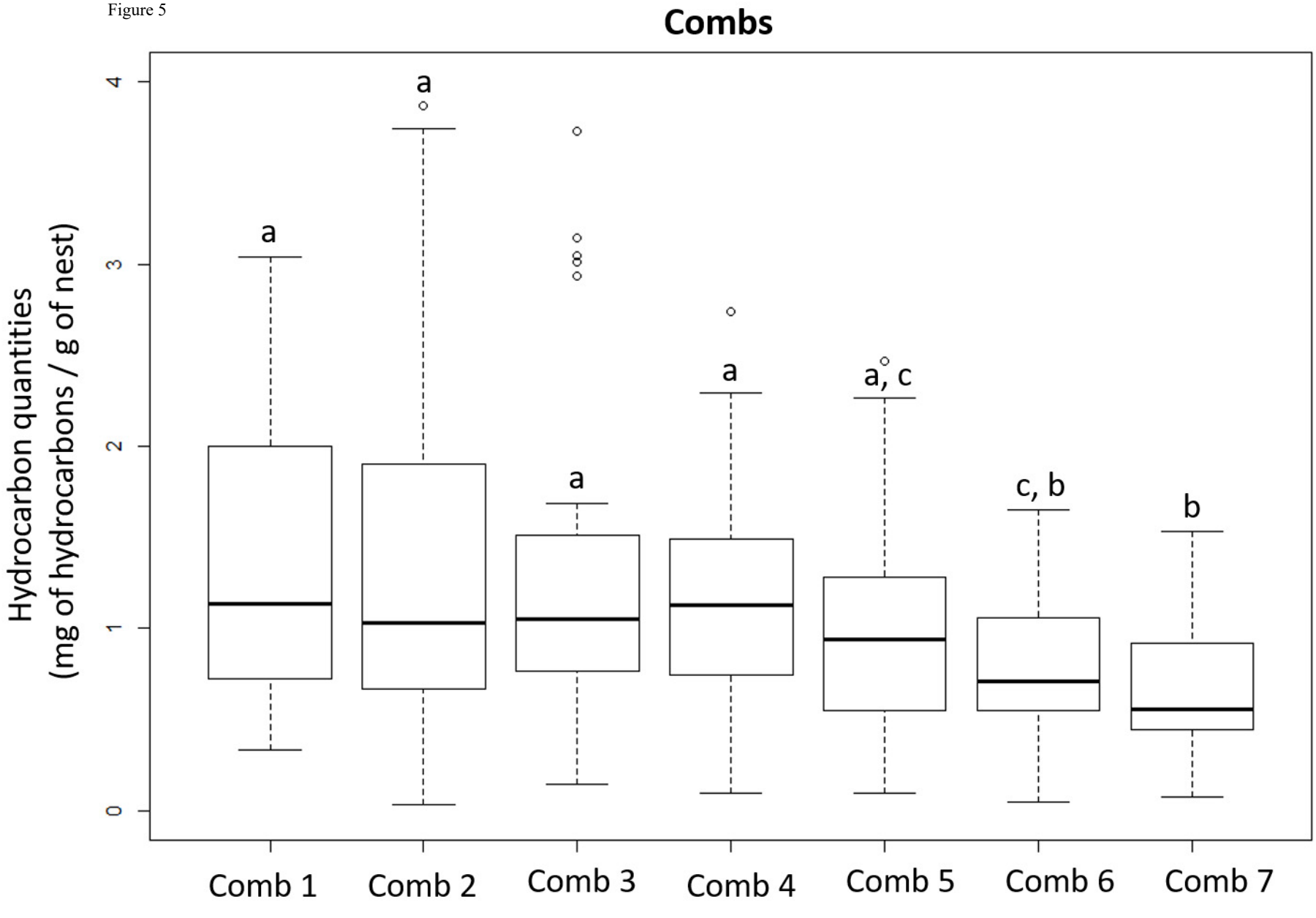
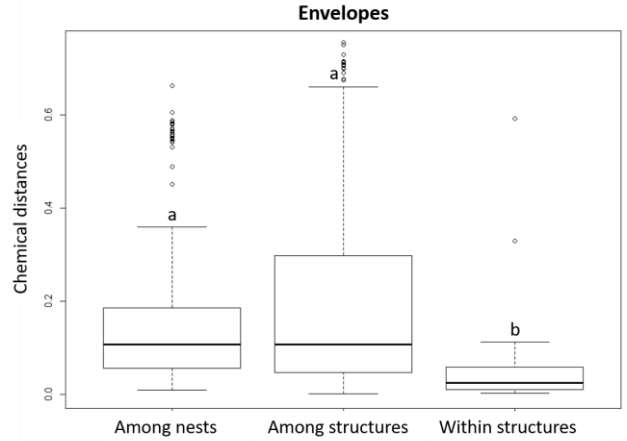
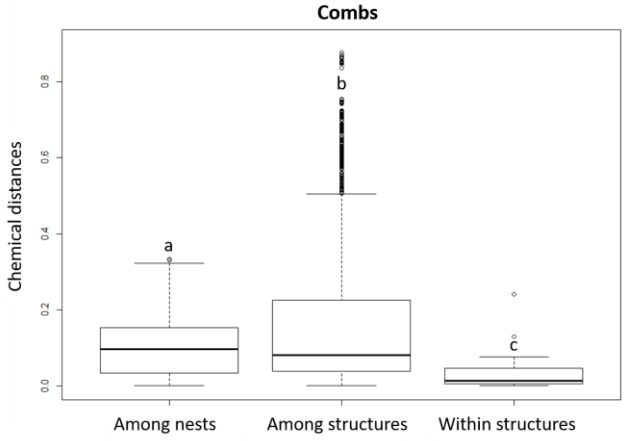

Combs
Hydrocarbon quantities
(mg of hydrocarbons / g of nest)
0
1
2
3
4
a
b
c
Center
Middle
Periphery

Figure 5


Combs
Hydrocarbon quantities
(mg of hydrocarbons / g of nest)
0
1
2
3
4
a
a
a
a
a, c
c, b
b
Comb 1
Comb 2
Comb 3
Comb 4
Comb 5
Comb 6
Comb 7


Envelopes
Chemical distances
0.0
0.2
0.4
0.6
a
a
b
Among nests
Among structures
Within structures


Combs
Chemical distances
0.0
0.2
0.4
0.6
0.8
a
b
c
Among nests
Among structures
Within structures

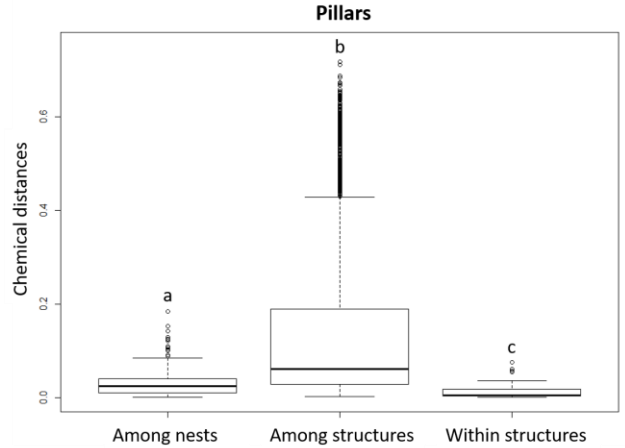
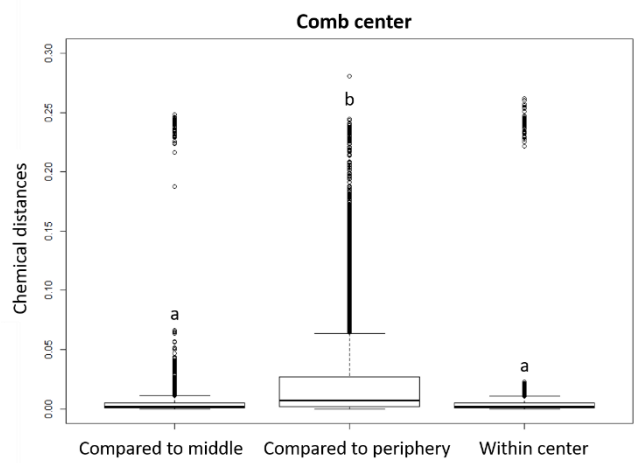
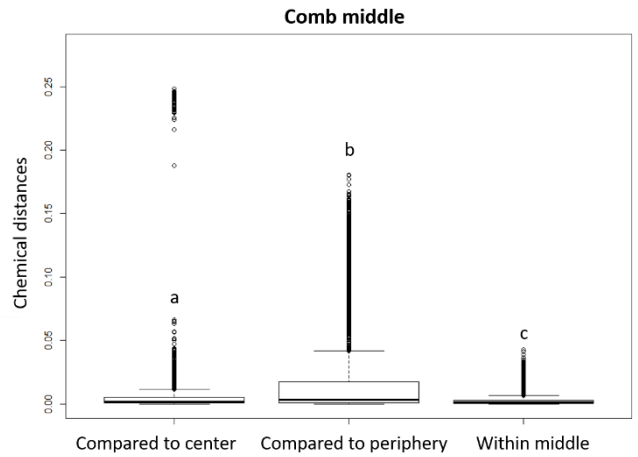

Pillars
Chemical distances
0.0
0.2
0.4
0.6
a
b
c
Among nests
Among structures
Within structures

Figure S3


Comb center
Chemical distances
0.00
0.05
0.10
0.15
0.20
0.25
0.30
a
b
a
Compared to middle
Compared to periphery
Within center


Comb middle
Chemical distances
0.00
0.05
0.10
0.15
0.20
0.25
a
b
c
Compared to center
Compared to periphery
Within middle

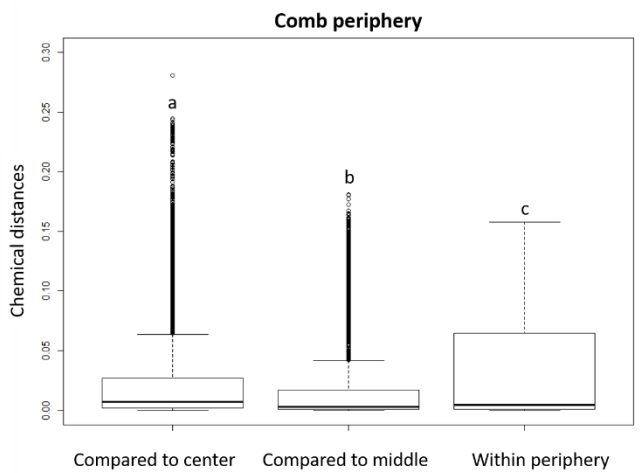
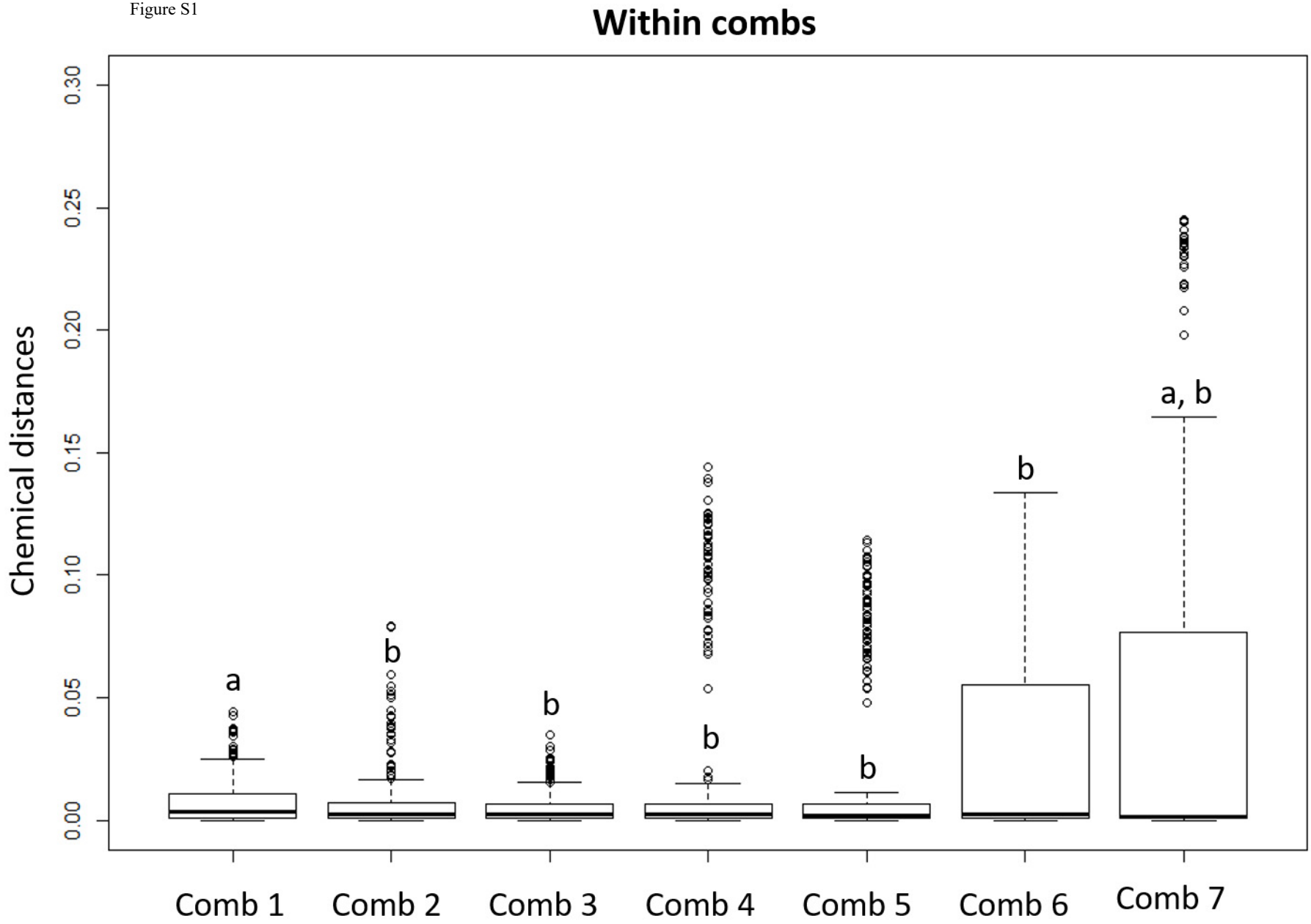

Comb periphery
Chemical distances
0.00
0.05
0.10
0.15
0.20
0.25
0.30
a
b
c
Compared to center
Compared to middle
Within periphery

Figure S2

Figure S1


Within combs
Chemical distances
0.00
0.05
0.10
0.15
0.20
0.25
0.30
a
b
b
b
b
b
a, b
Comb 1
Comb 2
Comb 3
Comb 4
Comb 5
Comb 6
Comb 7