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Cuticular hydrocarbons as a tool for the identification of insect species: Puparial cases from Sarcophagidae

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Abstract

The external surface of all insects is covered by a species-specific complex mixture of highly stable, very long chain cuticular hydrocarbons (CHCs). Gas chromatography coupled to mass spectrometry was used to identify CHCs from four species of Sarcophagidae, *Peckia* (*Peckia*) *chrysostoma*, *Peckia* (*Pattonella*) *intermutans*, *Sarcophaga* (*Liopygia*) *ruficornis* and *Sarcodexia* *lambens*. The identified CHCs were mostly a mixture of *n*-alkanes, monomethylalkanes and dimethylalkanes with linear chain lengths varying from 23 to 33 carbons. Only two alkenes were found in all four species. *S. lambens* had a composition of CHCs with linear chain lengths varying from C23 to C33, while the other three species linear chain lengths from 24 to 31 carbons. *n*-Heptacosane, *n*-nonacosane and 3-methylnonacosane, *n*-triacontane and *n*-hentriacontane occurred in all four species. The results show that these hydrocarbon profiles may be used for the taxonomic differentiation of insect species and are a useful additional tool for taxonomic classification, especially when only parts of the insect specimen are available.

Keywords

Cuticular hydrocarbons; Insect identification; Diptera; Sarcophagidae; Gas chromatography–mass spectrometry; Forensic entomology

1. Introduction

The occurrence and activity of arthropods in corpses are used to determine the post-mortem interval (interval between the actual death and the finding of the body – PMI), if the body has been moved, the form and cause of death and to associate suspects to the crime scene (Campobasso and Introna, 2001; Oliveira-Costa, 2007). Forensic entomology may be used to estimate PMI in criminal cases since the insects are usually the first to find a decomposing body. The blow flies (Calliphoridae) will lay eggs on the body shortly after death (first hours) (Catts and Goff, 1992). The age of the larvae and of the adults is the basis to determine the PMI. However, the blow fly larvae are usually present during the summer in corpses that are not buried and the succession of species found around and on the decomposing bodies vary with time. The sequence of succession is predictable to some extent as decomposition advances. The insects are attracted by the state of decomposition of the body and form complex communities that include necrophagous, predator and parasite species, as well as parasitoids. Besides the determination of when the death occurred, the study of the succession of the entomofauna may also be used to determine where it occurred. The identification of the species, the knowledge of its history, the duration of each stage in different temperatures, and other abiotic factors allow establishing the PMI with higher precision (Turchetto and Vanin, 2004).

Until recently, the evaluation of the PMI was based on succession tables of cadaveric entomofauna described in the 19th century (Jonston and Villeneuve, 1897; Mégnin, 1894). However, there are differences in the cadaveric fauna depending on the region (country, continent). Therefore, recent authors included changes related to geographical region, latitude, ecosystem, climate etc. (Turchetto and Vanin, 2004).

One of the difficulties in determining the PMI is the absence of insect specimens, since they have to be identified and their stage determined. Forensic experts collect all insect specimens (adults, immature stages, puparial cases and eggs) – on or around a dead body. Frequently only puparial cases are found. The chemical degradation of puparial cases is slow and they are often found near cadavers – sometimes even several years after death. The identification of puparial cases of forensic important species is a major problem since they are often too deteriorated by the mechanical activity of the adult emergence and, in this case, the identification using the normal taxonomical methods is very difficult. Even molecular techniques may not be used in most cases due to the natural decomposition of DNA, proteins and enzymes (Ye et al., 2007). Several authors have demonstrated the usefulness of cuticular hydrocarbons for identifying insect species, including parasitic wasps (Bernier et al., 1997), phlebotomines (Bejarano et al., 2003; Mahamat and Hassanali, 1998), anophelines (Anyanwu et al., 2000, 2001), culicids (Horne and Priestmann, 2002) and triatomines (Calderón-Fernández et al., 2005a,b). The external surface of all insects is covered by a layer of species-specific cuticular lipids. These lipids are often composed primarily of a complex mixture of hydrocarbons that restrict water loss and act as close range or contact pheromones. The cuticular hydrocarbons consist of *n*-alkanes, terminally and internally branched mono and polymethyl alkanes, and alkenes (Blomquist and Bagnères, 2010; Blomquist and Dillwith, 1985; Blomquist et al., 1987; Catts and Haskell, 1990; Drijfhout, 2010; Gibbs, 1998, 2002; Howard and Blomquist, 1982, 2005; Lockey, 1988, 1991). In 2007 it was observed that these differences along with some other properties may allow the cuticular hydrocarbon composition to be used to determine the weathering time of the exuviae and, also, the PMI with greater precision (Zhu et al., 2007). The same technique was used to determine the age and survivorship of three species of laboratory-reared Australasian mosquitoes (*Aedes aegypti* (Linnaeus, 1762), *Anopheles farauti* Laveran, 1902 and *Ochlerotatus vigilax* (Skuse, 1889)) (Blomquist and Bagnères, 2010).

The identification of puparial cases of different species of dipterans of forensic importance using morphological methodologies is difficult due to the few available features and to the partial destruction that occurs during adult emergence (Anyanwu et al., 2000). The aim of this paper is to use the technique of gas chromatography coupled to mass spectrometry (GC–MS) to study the hydrocarbon composition of the puparial cases of four species of dipterans from the Sarcophagidae family to determine if this data could be used for insect identification.

2. Materials and methods

2.1. Collection and maintenance of the insects

Colonies of the species *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830), *Peckia* (*Pattonella*) *intermutans* (Thomson, 1869), *Sarcodexia* *lambens* (Wiedemann, 1830) and *Sarcophaga* (*Liopygia*) *ruficornis* (Fabricius, 1794) were established and maintained in the laboratory (Medical and Forensic Entomology Sector, LTL, Oswaldo Cruz Institute, Oswaldo Cruz Foundation – FIOCRUZ – Rio de Janeiro, Brazil). The colonies were kept under laboratory conditions, in a climatic chamber with 27 ± 1 °C, $60 \pm 10\%$ Relative humidity and a 12 h photoperiod (12 h light/12 h dark). Each species was placed in cages separately.

The fly colonies were maintained in cubic cages (30 cm × 30 cm × 30 cm) made of a wooden frame closed with nylon fabric. One of the sides was closed with a sleeve-like fabric to facilitate changes of water and food and to avoid the escape of the flies during these proceedings. The eggs were transferred to a new diet (liver) where they hatched and the larvae developed.

Liver was divided in three equal parts (250 g) and offered to the larvae of all four species. After the larvae abandoned the liver, they were individually weighed and transferred to glass tubes and maintained under controlled conditions. One fourth of the test tubes were filled with vermiculite and closed with hydrophobic cotton plugs for the pupation, emergence of the adults and observation of morphological alterations. After the adult emergence the puparial cases were stored for the hydrocarbon extraction.

2.2. Hydrocarbon (HC) extraction

The hydrocarbon extractions were made in triplicates using 3 puparial cases of *P. (P.) chrysostoma*, *P. (P.) intermutans* and *S. (L.) ruficornis* and 6 of *S. lambens*. The difference in the number of puparial cases was due to the size of the insects (the first three species were much bigger than the fourth) and did not interfere in the final result.

The puparial cases were immersed twice (10 min + 1 min) in 1 mL of redistilled hexane in borosilicate glass vials of 17- by 50-mm, at room temperature. The hexane was transferred through a Florisil chromatography column (Florisil Adsorbent 60–100 mesh, Fischer-Chemicals) onto borosilicate glass vials of 12- by 32-mm. After column chromatography, the CHCs were concentrated using a nitrogen flow. They were resuspended in 10 µL of redistilled hexane for the GC–MS analysis.

2.3. GC–MS analysis

Aliquots (1 µL) were analyzed by GC–MS using the splitless injection mode onto a capillary DB-5 column (30 m length, 0.25 mm diameter, 0.25 µm thick film). The initial temperature of the oven was 150 °C and increased to a final temperature of 320 °C, at a 5 °C/min ramp. The temperatures of the injector and the MS detector were 290 and 325 °C, respectively.

Analyses were performed on a Thermo-Finnigan Trace GC with Polaris Q Mass Spectrometer (GC–MS) in the Proteomics Center of Nevada, UNR, Reno, NV, USA. Helium was used as the carrier gas.

The GC–MS yielded qualitative results and was used to identify components. Cuticular hydrocarbons with carbon chain lengths of 21 carbons or more were selected to be analyzed since these are the ones usually present in insects. The analyses were made in triplicate for each species. The analyzed peaks were numbered according to their retention times. The relative abundance was calculated by computing the area of each peak which produced a percentage of the total peak area of all components in the sample for each species.

The identification of the hydrocarbons was made according to the EI mass spectra as described (Blomquist et al., 1987). The positions of the double bonds and of the methyl branches in the alkenes were not determined due to small sample size. Several peaks were mixtures of two or more compounds that eluted with the same retention times and equivalent chain lengths and therefore were listed as “mixture of” in Table 1. The nomenclature used to list hydrocarbons in Table 1 was Cxx to describe the total number of carbons in the corresponding hydrocarbon component; the location of methyl groups is indicated by x-Me for monomethylalkanes and x,y-dime for dimethylalkanes when one or two methyl groups are located in the molecule, respectively. For alkenes the nomenclature was Cxx:y depending on whether the compound had one or more double bonds in the chain. The cuticular hydrocarbons are listed in Table 1 according to their equivalent chain length (ECL).

2.4. Data analysis

Data analysis from the four species was made to determine if it is possible to use the hydrocarbon profiles to discriminate insect species. Data was normalized using the GraphPad Prism 6 software. For the normalization, zero percent was defined as the smallest value in the sets of data and 100% as the highest value. The normalization was performed by the software automatically. The Bray–Curtis similarity index (Bray and Curtis, 1957; Anu and Sabu, 2006) of the normalized data was computed among all possible pairs of species and the matrix was submitted to cluster analysis (PAST software, version 2.17) to draw a hierarchical tree. Bray–Curtis similarity index analyzes the presence or absence of each hydrocarbon and the variation of their relative abundances.

3. Results and discussion

The comparison of the hydrocarbon profiles of *P. (P.) chrysostoma*, *P. (P.) intermutans*, *S. (L.) ruficornis* and *S. lambens* are shown in Fig. 1.

Table 1 lists the compounds found in the four species. The analyses demonstrated that the hydrocarbons from the four species are mostly a mixture of *n*-alkanes, monomethyl alkanes and dimethyl alkanes. Only two alkenes were found. As shown in Table 1, *S. lambens* had a mixture of hydrocarbons with linear chain lengths varying from C23 to C33 while the other three species had from 24 to 31 carbons. x-Methyltetracosane, *n*-heptacosane, 3-methylheptacosane, *n*-nonacosane, *n*-triacontane and *n*-hentriacontane occurred in all species. Odd-numbered hydrocarbons were predominant: 71.43% for *P. (P.) chrysostoma*, 62.07% for *P. (P.) intermutans*, 79.17% for *S. (L.) ruficornis* and 80.5% for *S. lambens*.

Fifty three hydrocarbons were identified in 46 peaks and all four species have a distinct overall composition. *P. (P.) chrysostoma* major compounds are *n*-nonacosane (23.51%), 3-methylnonacosane (15.23%) and a mixture of 11-, 13- and 15-methylnonacosane (14.19%). *P. (P.) intermutans* major compounds are *n*-nonacosane (22.20%), 3-methylnonacosane

(15.56%) and a mixture of 13- and 15-methylnonacosane (11.28%). *S. lambens* has the following major compounds: 3-methylheptacosane (17.45%), *n*-heptacosane (15.48%) and *n*-nonacosane (11.64%); and the major compounds of *S. (L.) ruficornis* are 3-methylnonacosane (25.13%), *n*-nonacosane (15.43%) and a mixture of 13- and 15-methylnonacosane (15.20%). The *n*-alkanes represented 25, 20.69, 33.33 and 19.05% of the compounds of *P. (P.) chrysostoma*, *P. (P.) intermutans*, *S. lambens* and *S. (L.) ruficornis*, respectively. Methyl branched alkanes were the majority of the compounds for all species: 71.43, 75.86, 58.33 and 71.43% (*P. (P.) chrysostoma*, *P. (P.) intermutans*, *S. lambens* and *S. (L.) ruficornis*, respectively). Monomethyl alkanes represented 80, 86.36, 85.71 and 73.33%, while dimethyl alkanes represented only 20, 13.64, 14.28 and 26.67%, of the total methyl branched alkanes of *P. (P.) chrysostoma*, *P. (P.) intermutans*, *S. lambens* and *S. (L.) ruficornis*, respectively; all four species had one alkene, a monomethyl branched alkene (x-methyltetracosene). Squalene (C30:6) was found in the extract of *S. lambens* and *S. (L.) ruficornis*. As insects do not make squalene, it is most likely taken up while feeding on flesh; it was, however, taken in account in the relative abundance calculations.

The hierarchical classification (Fig. 2) calculated by cluster analysis (Cophenetic Correlation Coefficient = 0.9236) of the Bray–Curtis similarity index was obtained by comparing the hydrocarbon profiles of the four species. With this analysis it can be observed that *S. lambens* is dissimilar from the other three species, but more related to *S. ruficornis*, while *P. (P.) chrysostoma* and *P. (P.) intermutans* are closely related to each other. *S. lambens*, however, was not classified as subspecies of the genus *Peckia* Robineau-Desvoidy as stated in the recently published revision of this genus (Buenaventura and Pape, 2013). These results, however, are in accordance with the classical taxonomy that classified *Sarcodexia* as a separate genus (Pape, 1996).

The identification of the four species according to its hydrocarbon composition demonstrates that this is a highly reliable tool in insect taxonomy and play an important role in chemotaxonomy (Kaib et al., 1991; Kather and Martin, 2012; Nowbahari et al., 1990). Very few papers have been published on hydrocarbons from necrophagous insects (Catts and Haskell, 1990; Roux et al., 2008; Ye et al., 2007; Zhu et al., 2006). In the last few years it is becoming increasingly clear that the use of hydrocarbons is a valuable tool for forensic entomology. It is a fast, reliable and fairly affordable technique. It can be used as an alternative method when the taxonomical identification of the insect is not feasible due to its damaged condition or if its DNA is too degraded. DNA inside puparial cases is very small in the amount and becomes degraded after some time exposed to the environment.

Classical taxonomical characters for the identification of puparial cases of *Chrysomya megacephala* (Fabricius, 1754), *Chrysomya putoria* (Wiedemann, 1818) and *Cochliomyia macellaria* (Fabricius, 1775) are not particularly useful due to the availability of only three useful morphological characters: the tubercles in the perispiracular region, the texture of the puparial surface and the distances between the peritremas in the posterior spiracular region (Amorim and Ribeiro, 2001). CHCs became a useful tool for the distinction of species complex or populations of several insects including cockroaches (Carlson and Brenner, 1988; Everaerts et al., 1997), mosquitoes (Horne and Priestmann, 2002; Kruger et al., 1991; Milligan et al., 1986; Phillips et al., 1990), a fly species, *Phormia regina* Meigen 1826 (Byrne et al., 1995), tabanids (Sakolsky et al., 1999) and termites (Howard et al., 1988; Takematsu and Yamaoka, 1999; Thorne et al., 1994). Sometimes the differences are small but still important demonstrating the usefulness of this technique.

The present study shows that the differentiation of species of Sarcophagidae is possible using their cuticular hydrocarbons. There has been only one study published to date using this technique for the same purpose with puparial cases of forensic important flies (Ye et al.,

2007). The authors were able to differentiate six species of necrophagous flies from China: *Aldrichina graham* (Aldrich, 1930), *C. megacephala*, *Lucilia sericata* Meigen, 1826, *Achoetandrus rufifacies* (Macquart, 1843), *Boettcherisca peregrina* Robineau-Desvoidy, 1830 and *Parasarcophaga crassipalpis* Macquart, 1839.

Environmental factors have a limited effect on cuticular hydrocarbons (Page et al., 1990). Adult *P. regina* fed with sugar or protein presented no differences in diet-, age-, or sex-specific cuticular hydrocarbons (Stoffolano et al., 1997). Several other authors, however, reported that the hydrocarbon composition may vary due to geographical differences, diet and environmental temperature (Desena et al., 1999a,b; Liang and Silverman, 2000; Milligan et al., 1986). In the present study, Squalene was the only foreign hydrocarbon present in the puparial cases.

Several authors have been using the Bray–Curtis similarity index for cluster analysis of ecological data (Anu and Sabu, 2006; Singh, 2008; Majumder et al., 2012). It has been demonstrated that the analysis using Bray–Curtis distances (Bray and Curtis, 1957) is an appropriate tool for the evaluation of the discrimination of insect species using hydrocarbon profiles.

The insects used in our study were maintained in similar conditions in the laboratory, however we do not know if similar results may be obtained with flies collected directly from the field. Future studies on this subject are to be performed to confirm those obtained with laboratory reared insects.

4. Conclusion

Mendonça et al. (2010, 2012a,b) used scanning electron microscopy for the description of morphological features present in the immature stages of Calliphoridae (*Chrysomya albiceps* (Wiedemann, 1819), *C. megacephala* and *C. putoria*) that cannot be observed clearly with the aid of light microscopy. These features in addition to classical taxonomy and the HC profiles are tools that may help clarify identification problems of cryptic insect species.

These data may be used for taxonomic differentiation of necrophagous insect species and are of extreme importance for forensic entomology especially in the determination of the postmortem interval (PMI). Hydrocarbons are very useful tools for the identification of insects since even broken puparial cases and parts of insects may be used for the analysis; these highly stable molecules can be used also for older specimens. This method is simple, feasible and cost-effective and even forensic specialists with little entomological knowledge may use this technique for taxonomical purposes.

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References

- Amorim JA, Ribeiro OB. Distinction among the puparia of three blowfly species (Diptera: Calliphoridae) frequently found on unburied corpses. *Memórias do Instituto Oswaldo Cruz*. 2001; 96:781–784. [PubMed: 11562702]
- Anu A, Sabu TK. Biodiversity analysis of forest litter ant assemblages in the Wayanad region of Western Ghats using taxonomic and conventional diversity measures. *Journal of Insect Science*. 2006; 7:1–13.
- Anyanwu GI, Molyneux DH, Phillips A. Variation in cuticular hydrocarbons among strains of the *Anopheles gambiae sensu stricto* by analysis of cuticular hydrocarbons using gas liquid chromatography of larvae. *Memórias do Instituto Oswaldo Cruz*. 2000; 95:295–300. [PubMed: 10800186]
- Anyanwu GI, Molyneux DH, Priestman A. Cuticular-hydrocarbon discrimination between *Anopheles gambiae* s.s. and *An. Arabiensis* larval karyotypes. *Annals of Tropical Medicine and Parasitology*. 2001; 95:843–852. [PubMed: 11784439]
- Bejarano EE, Rojas W, Uribe S, Vélez ID. Sistemática de espécies de *Lutzomyia* del grupo verrucarum Theodor, 1965 (Diptera: Psychodidae). *Biomedica*. 2003; 23:87–102. [PubMed: 12696402]
- Bernier UR, Carlson DA, Geden CJ. Gas chromatography/mass spectrometry analysis of the cuticular hydrocarbons from parasitic wasps of the genus *Muscidifurax*. *Journal of the American Society for Mass Spectrometry*. 1997; 9:320–332.
- Blomquist, GJ.; Bagnères, A-G. *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*. Cambridge University Press; Cambridge, UK: 2010. p. 492
- Blomquist, GJ.; Dillwith, JW. Cuticular lipids. In: Kerkutt, GA.; Gilberts, LL., editors. *Comprehensive Insect Physiology Biochemistry and Pharmacology*. Vol. 3. Pergamon Press; Oxford: 1985. p. 117-154.
- Blomquist GJ, Nelson DR, Renobales MD. Chemistry, biochemistry, and physiology of insect cuticular lipids. *Archives of Insect Biochemistry and Physiology*. 1987; 6:227–265.
- Bray JR, Curtis JT. An ordination of the upland forest communities of Southern Wisconsin. *Ecological Monographs*. 1957; 27:325–349.
- Buenaventura E, Pape T. Revision of the new world genus *Peckia* Robineau-Desvoidy (Diptera: Sarcophagidae). *Zootaxa*. 2013; 3622:1–87.
- Byrne AL, Camann MA, Cyr TL, Catts EP, Espelie KE. Forensic implication of biochemical differences among geographic population of the black blow fly, *Phormia regina* (Meigen). *Journal of Forensic Sciences*. 1995; 40:372–377. [PubMed: 7782742]
- Calderón-Fernández G, Juárez MP, Monroy MC, Menes M, Bustamante DM, Mijailovsky S. Intraspecific variability in *Triatoma dimidiata* (Hemiptera: Reduviidae) populations from Guatemala based on chemical and morphometric analyses. *Journal of Medical Entomology*. 2005a; 42:29–35. [PubMed: 15691005]
- Calderón-Fernández G, Juárez MP, Ramsey J, Salazar-Schettino PM, Monroy MC, Ordoñez R, Cabrera M. Cuticular hydrocarbon variability among *Triatoma dimidiata* (Hemiptera: Reduviidae) populations from Mexico and Guatemala. *Journal of Medical Entomology*. 2005b; 42:780–788. [PubMed: 16363161]
- Campobasso CP, Introna F. The forensic entomologist in the context of the forensic pathologist's role. *Forensic Science International*. 2001; 120:132–139. [PubMed: 11457621]
- Carlson DA, Brenner RJ. Hydrocarbon-based discrimination of three North American *Blattella* cockroach species (Orthoptera: Blattellidae) using gas chromatography. *Annals of the Entomological Society of America*. 1988; 81:711–723.
- Catts EP, Goff ML. Forensic entomology in criminal investigations. *Annual Review of Entomology*. 1992; 37:253–272.
- Catts, EP.; Haskell, NH. *Entomology and Death: A Procedural Guide*. Joyce's Print Shop; Clemson, SC: 1990.
- Desena ML, Clark JM, Edman JD, Symington SB, Scott TW, Clark GG, Peters TM. Potential for aging female *Aedes aegypti* (Diptera: Culicidae) by gas chromatographic analysis of cuticular

- hydrocarbons, including a field evaluation. *Journal of Medical Entomology*. 1999a; 36:811–823. [PubMed: 10593085]
- Desena ML, Edman JD, Clark JM, Symington SB, Scott TW. *Aedes aegypti* (Diptera: Culicidae) age determination by cuticular hydrocarbon analysis of female legs. *Journal of Medical Entomology*. 1999b; 36:824–830. [PubMed: 10593086]
- Drijfhout, FK. Cuticular hydrocarbons: a new tool in forensic entomology?. In: Amendt, J.; Lee Goff, M.; Campobasso, CP.; Grassberger, M., editors. *Current Concepts in Forensic Entomology*. Springer Press; Dordrecht Heidelberg London New York: 2010. p. 179-203.
- Everaerts C, Farine JP, Brossut R. Changes of species specific cuticular hydrocarbon profiles in the cockroaches *Nauphoeta cinerea* and *Leucophaea maderae* reared in heterospecific groups. *Entomologia Experimentalis et Applicata*. 1997; 85:145–150.
- Gibbs AG. Water-proofing properties of cuticular lipids. *American Zoologist*. 1998; 38:471–482.
- Gibbs AG. Lipid melting and cuticular permeability: new insights into an old problem. *Journal of Insect Physiology*. 2002; 48:391–400. [PubMed: 12770088]
- Horne GL, Priestmann AA. The chemical characterization of the epicuticular hydrocarbons of *Aedes aegypti* (Diptera: Culicidae). *Bulletin of Entomological Research*. 2002; 92:287–294. [PubMed: 12191436]
- Howard RW, Blomquist GJ. Chemical ecology and biochemistry of insect hydrocarbons. *Annual Review of Entomology*. 1982; 27:149–172.
- Howard RW, Blomquist GJ. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology*. 2005; 50:371–393.
- Howard RW, Thorne BL, Levings SC, McDaniel CA. Cuticular hydrocarbons as chemotaxonomic characters for *Nasutitermes corniger* (Motschulsky) and *N. ephratae* (Holmgren) (Isoptera: Termitidae). *Annals of the Entomological Society of America*. 1988; 81:395–399.
- Jonston W, Villeneuve G. On the medico-legal application of entomology. *Montreal Medical Journal*. 1897; 26:81–90.
- Kaib M, Brandi R, Bagine RKN. Cuticular hydrocarbon profiles: a valuable tool in termite taxonomy. *Naturwissenschaften*. 1991; 78:176–179.
- Kather R, Martin SJ. Cuticular hydrocarbon profiles as a taxonomic tool: advantages, limitations and technical aspects. *Physiological Entomology*. 2012; 37:25–32.
- Kruger EL, Pappas CD, Howard R. Cuticular hydrocarbon geographic variation among seven North America populations of *Aedes albopictus* (Diptera: Culicidae). *Journal of Medical Entomology*. 1991; 28:859–864. [PubMed: 1770522]
- Liang D, Silverman J. You are what you eat”: diet modifies cuticular hydrocarbons and nestmate recognition in the Argentine ant *Linepithema humile*. *Naturwissenschaften*. 2000; 87:412–416. [PubMed: 11091966]
- Lockey KH. Lipids of the insect cuticle: origin, composition and function. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*. 1988; 89:595–645.
- Lockey KH. Insect hydrocarbon classes: implications for chemotaxonomy. *Insect Biochemistry*. 1991; 21:91–97.
- Mahamat H, Hassanali A. Cuticular hydrocarbon composition analysis for taxonomic differentiation of phlebotomine sandfly species (Diptera: Psychodidae) in Kenya. *Journal of Medical Entomology*. 1998; 35:778–781. [PubMed: 9775608]
- Majumder J, Lodh R, Agarwala BK. Variation in butterfly diversity and unique species richness along different habitats in Trishna Wildlife Sanctuary, Tripura, northeast India. *Check List*. 2012; 8:432–436.
- Méglin, P. Application de l'entomologie a la médecine légale, *Encyclopedie scientifique des Aides-Memoirés*. Marson, Paris: Gauthiers-Villards, Paris; 1894. La faune des cadavres.
- Mendonça PM, Dos Santos-Mallet JR, Queiroz MM. Ultramorphological characteristics of immature stages of *Chrysomya albiceps* (Wiedemann 1819) (Diptera: Calliphoridae), a fly specie of forensic importance. *Microscopy Research and Technique*. 2010; 73:779–784. [PubMed: 20063314]
- Mendonça PM, Dos Santos-Mallet JR, De Carvalho Queiroz MM. Ultrastructure of immature stages of the blowfly *Chrysomya putoria* (Wiedemann, 1818) (Diptera: Calliphoridae). *Microscopy Research and Technique*. 2012a; 75:206–211. [PubMed: 21761503]

- Mendonça PM, dos Santos-Mallet JR, De Carvalho Queiroz MM. Ultrastructure of larvae and puparia of the blowfly *Chrysomya megacephala* (Diptera: Calliphoridae). *Microscopy Research and Technique*. 2012b; 75:935–939. [PubMed: 22362589]
- Milligan PJM, Phillips A, Molyneux DH, Subbarao SK, White GB. Differentiation of *Anopheles licifacies* Giles (Diptera: Culicidae) sibling species by analysis of cuticular components. *Bulletin of Entomological Research*. 1986; 76:529–537.
- Nowbahari E, Lenoir A, Clément JL, Lange C, Bagnères AG, Joulie C. Individual, geographical, and experimental variation of cuticular hydrocarbons of the ant *Cataglyphis cursor* (Hymenoptera: Formicidae): their use in nest and subspecies recognition. *Biochemical Systematics and Ecology*. 1990; 18:63–73.
- Oliveira-Costa, J. A entomologia Forense e suas aplicações. In: Oliveira-Costa, J., editor. *Entomologia Forense. Quando os insetos são vestígios*. Millennium Editora Ltda; Campinas, SP: 2007. p. 39-50.
- Page M, Nelson LJ, Haverty MI, Blomquist GJ. Cuticular hydrocarbons as chemotaxonomic characters for bark beetles: *Dendroctonus ponderosae*, *D. jeffreyi*, *D. brevicornis*, and *D. frontalis* (Coleoptera: Scolytidae). *Annals of the Entomological Society of America*. 1990; 83:892–901.
- Pape, T. *Memoirs on Entomology, International Series*. Associated Publishers; Gainesville, FL, USA: 1996. Catalogue of the Sarcophagidae of the world (Insecta: Calyptratae); p. 560
- Phillips A, Sabatini A, Milligan PJM, Boccolini D, Broomfield G, Molyneux DH. The *Anopheles maculipennis* complex (Diptera: Culicidae): comparison of the cuticular hydrocarbon profiles determined in adults of five Palaearctic species. *Bulletin of Entomological Research*. 1990; 80:459–464.
- Roux O, Gers C, Legal L. Ontogenetic study of three Calliphoridae of forensic importance through cuticular hydrocarbon analysis. *Medical and Veterinary Entomology*. 2008; 22:309–317. [PubMed: 19120957]
- Sakolsky G, Carlson DA, Sutton BD, Stoffolano JG. Detection of cryptic species in the *Tabanus nigrovittatus* (Diptera: Tabanidae) complex in Massachusetts by morphometric and cuticular hydrocarbon analysis. *Journal of Medical Entomology*. 1999; 36:610–613. [PubMed: 10534956]
- Singh, W. Master of Science Thesis. University of Iceland; Reykjavík, Iceland: 2008. Robustness of three hierarchical agglomerative clustering techniques for ecological data. RH-12-2008
- Stoffolano JG, Schaubert E, Yin CM, Tillman JA, Blomquist GJ. Cuticular hydrocarbons and their role in copulatory behavior in *Phormia regina* (Meigen). *Journal of Insect Physiology*. 1997; 43:1065–1076. [PubMed: 12770478]
- Takematsu Y, Yamaoka R. Cuticular hydrocarbons of *Reticulitermes* (Isoptera: Rhinotermitidae) in Japan and neighboring countries as chemotaxonomic characters. *Applied Entomology and Zoology*. 1999; 34:179–188.
- Thorne BL, Haverty MI, Collins MS. Taxonomy and biogeography of *Nasutitermes acajutlae* and *N. nigriceps* (Isoptera: Termitidae) in the Caribbean and Central America. *Annals of the Entomological Society of America*. 1994; 87:762–770.
- Turchetto M, Vanin S. Forensic entomology and climatic change. *Forensic Science International*. 2004; 146S:S207–S209. [PubMed: 15639577]
- Ye G, Li K, Zhu J, Zhu G, Hu C. Cuticular hydrocarbon composition in pupal exuviae for taxonomic differentiation of six necrophagous flies. *Journal of Medical Entomology*. 2007; 44:450–456. [PubMed: 17547230]
- Zhu GH, Ye GY, Hu C, Xu XH, Li K. Development changes of cuticular hydrocarbons in *Chrysomya rufifacies* larvae: potential for determining larval ages. *Medical and Veterinary Entomology*. 2006; 20:438–444. [PubMed: 17199755]
- Zhu GH, Xu XH, Yu XJ, Zhang Y, Wang JF. Puparial case hydrocarbons of *Chrysomya megacephala* as an indicator of the postmortem interval. *Forensic Science International*. 2007; 169:1–5. [PubMed: 16930899]

Fig. 1. Comparison of the gas chromatogram profiles from puparial cases of *Peckia (Peckia) chrysostoma*, *Peckia (Pattonella) intermutans*, *Sarcodexia lambens* and *Sarcophaga (Liopygia) ruficornis*. Numbers above peaks represent the peak numbers as listed in Table 1.

Fig. 2. Hierarchical tree made by clustering of Bray–Curtis similarity index among hydrocarbon profiles analyzed by GC–MS. Representation of the general phylogenetic relationships among *Peckia (Peckia) chrysostoma*, *Peckia (Pattonella) intermutans*, *Sarcodexia lambens* and *Sarcophaga (Liopygia) ruficornis*.

Mean and standard deviations of the relative abundances of cuticular hydrocarbons (CHCs) from puparial cases of *Peckia* (*Peckia*) *chrysostoma*, *Peckia* (*Pattonella*) *ntermutans*, *Sarcodexia* *lambens* and *Sarcophaga* (*Liopygia*) *ruficornis*.

Table 1

Peak #	Hydrocarbon (CHC)	ECL ^a	Relative abundance (%) ^b			
			<i>P. (P.) chrysostoma</i>	<i>P. (P.) intermutans</i>	<i>S. lambens</i>	<i>S. (L.) ruficornis</i>
1	<i>n</i> -C23	23.0	nd ^c	nd	5.47 ± 0.89	nd
2	Mixture of 7-,9- and 11-MeC23	23.4	nd	nd	2.06 ± 0.49	nd
3	<i>x</i> -MeC24:1	24.1	0.73 ± 0.49	0.45 ± 0.34	0.53 ± 0.08	1.03 ± 0.81
4	<i>n</i> -C25	25.0	0.51 ± 0.49	0.29 ± 0.24	1.82 ± 0.88	nd
5	Mixture of 11- and 13-MeC25	25.3	nd	nd	9.60 ± 2.08	nd
6	<i>n</i> -C26	26.0	0.36 ± 0.33	nd	nd	nd
7	<i>n</i> -C27	27.0	12.70 ± 2.84	7.66 ± 4.69	15.48 ± 2.63	5.25 ± 0.18
8	13-MeC27	27.3	nd	nd	10.64 ± 3.68	1.81 ± 0.93
9	11- MeC27	27.3	nd	nd	nd	0.47 ± 0.14
10	Mixture of 11- and 13- MeC27	27.3	3.55 ± 1.73	2.38 ± 1.09	nd	nd
11	4- MeC27	27.6	1.09 ± 0.71	nd	nd	nd
12	11,17-DimeC27	27.6	nd	nd	nd	0.61 ± 0.37
13	11,15-DimeC27	27.6	nd	0.80 ± 0.35	0.91 ± 0.19	nd
14	3-MeC27	27.7	6.66 ± 1.62	10.98 ± 1.89	17.45 ± 5.23	4.16 ± 0.75
15	<i>n</i> -C28	28.0	2.49 ± 0.06	2.10 ± 0.39	0.72 ± 0.09	nd
16	C30:6 (Squalene)	28.1	nd	nd	1.06 ± 0.89	4.57 ± 2.66
17	13-MeC28	28.3	0.76 ± 0.25	nd	0.53 ± 0.09	nd
18	Mixture of 11-,13- and 15-MeC28	28.3	nd	0.46 ± 0.20	nd	nd
19	6,15-Dime C28	28.3	nd	nd	nd	0.93 ± 0.17
20	4-MeC28	28.6	1.07 ± 0.54	2.00 ± 0.70	nd	nd
21	2-MeC28	28.6	0.56 ± 0.03	nd	nd	nd
22	3-MeC28	28.7	nd	0.41 ± 0.10	nd	nd
23	<i>n</i> -C29	29.0	23.51 ± 1.24	22.20 ± 4.52	11.64 ± 2.11	15.43 ± 4.24
24	Mixture of 13- and 15-MeC29	29.3	nd	11.28 ± 4.06	nd	15.20 ± 2.12
25	Mixture of 11- and 13-MeC29	29.3	nd	nd	4.79 ± 1.68	nd
26	Mixture of 11-,13- and 15-MeC29	29.3	14.19 ± 6.99	nd	nd	nd

Peak #	Hydrocarbon (CHC)	ECL ^a	Relative abundance (%) ^b			
			<i>P. (P.) chrysostoma</i>	<i>P. (P.) intermutans</i>	<i>S. lambens</i>	<i>S. (L.) ruficornis</i>
27	7-MeC29	29.4	nd	nd	nd	6.64 ± 1.36
28	5-MeC29	29.5	1.72 ± 0.55	nd	nd	2.96 ± 0.77
29	Mixture of 5- and 7-MeC29	29.5	nd	2.39 ± 1.02	nd	nd
30	11,17-DimeC29	29.6	nd	3.92 ± 2.16	nd	nd
31	13,19- DimeC29	29.6	nd	nd	nd	5.01 ± 0.61
32	11,15-DimeC29	29.6	nd	nd	nd	1.56 ± 0.52
33	Mixture of 11,15- and 11,17-DimeC29	29.6	4.82 ± 2.00	nd	nd	nd
34	3-MeC29	29.7	15.23 ± 3.15	15.56 ± 7.31	7.05 ± 1.30	25.13 ± 2.63
35	<i>n</i> -C30	30.0	2.65 ± 0.43	1.81 ± 0.99	0.75 ± 0.49	3.20 ± 0.27
36	13-MeC30	30.3	0.65 ± 0.06	nd	nd	nd
37	Mixture of 12-,13-,14- and 15-MeC30	30.3	nd	0.71 ± 0.07	nd	nd
38	<i>n</i> -C31	31.0	2.31 ± 0.74	1.46 ± 0.50	5.35 ± 3.04	4.26 ± 2.52
39	<i>x,y</i> -DimeC31	31.1	nd	nd	0.61 ± 0.03	nd
40	15-MeC31	31.3	nd	nd	0.83 ± 0.70	nd
41	Mixture of 13- and 15- MeC31	31.3	4.54 ± 0.06	nd	nd	nd
42	Mixture of 11-,13- and 15-MeC31	31.3	nd	7.53 ± 1.98	nd	1.81 ± 0.24
43	11,17-DimeC31	31.5	nd	5.12 ± 1.42	nd	nd
44	Mixture of 11,17-, 11,19- and 13,17- DimeC31	31.5	2.64 ± 0.06	nd	nd	nd
45	3-MeC31	31.7	0.53 ± 0.26	nd	0.35 ± 0.10	nd
46	<i>n</i> -C33	33.0	nd	nd	0.36 ± 0.03	nd

^aECL – Equivalent chain length.

^bThe relative abundance of each peak was calculated in relation to the total peak area of each sample (means and standard deviations of triplicates).

^cnd – not detected.