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The Attractivity of the Head Louse, *Pediculus humanus capitis* (Pediculidae: Anoplura) to Isolated Compounds and Blends of Human Skin

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Abstract

Human head lice *Pediculus humanus capitis* De Geer (Phthiraptera: Pediculidae) are ectoparasites that cause pediculosis, a global scale disease mainly found in school-age children. Previous works from our laboratory found nonanal, sulcatone, and geranylacetone as the main human scalp volatile components, and individually evaluated their attraction to head lice using an olfactometer. In this work, we compared how their blends at different concentrations attract head lice, and how their blended effect compares to the effect of isolated compounds. At the concentrations evaluated, individual components did not show attraction towards head lice, but a ternary mixture of them was attractive. Moreover, a solvent extract from the human head scalp was analyzed by GC-MS, finding that tetradecanoic acid, palmitic acid, oleic acid, palmitoleic acid, and squalene are the most abundant components. Attraction to these individual compounds at natural concentrations was tested by bioassays in a circular experimental arena. No attraction was observed when the components were tested individually, but when they were evaluated as a blend they attracted head lice. This work presents new information about how chemical signals are attractive at certain concentrations and proportions. This information could be used to better understand communication mechanisms in head lice and for the development of louse repellents.

Key words: head louse, head scalp compound, insect attraction

Human lice, commonly known as sucking lice, are hemimetabolous insects that belong to the suborder Anoplura (order: Phthiraptera, family: Pediculidae). They pierce the skin of their human hosts and feed on their blood, which is their only diet (Durden and Musser 1994). Pediculosis is a widespread disease that causes skin irritation and possible secondary infections (Toloza et al. 2009). Head lice infestation is highly prevalent in many societies, especially among school children, and pediculosis has been scientifically studied for centuries due to its close relationship to humans. Despite this, many facts about how human lice are attracted to their host are still unknown.

Head lice transmission in human hosts was recently analyzed by Heukelbach et al. (2017). These authors observed that when head lice were accidentally displaced onto clothes of people while screening children for pediculosis, they invariably started to climb towards the head. This work proves that head lice move against the gravitational pull. Gravity may be one of several factors important for orientation of head lice. The studies also indicated that surface anatomical factors of the hand and forearm, such as the presence of hair and its direction of growth, may be important in orientation. These authors also suggested that, in addition to head-to-head and fomite transmission, head-to-body transmission may occur in some circumstances, followed by movement of lice from one part of the body to the head of the new host.

Human skin contains up to 400 chemical compounds (Penn et al. 2007, Gallagher et al. 2008), some of which are used by blood-feeding insects in their search for blood. Chemical signals are also involved in the interaction between lice and humans. Wigglesworth (1941) studied attraction mechanisms of body lice (*Pediculus humanus humanus)* in one of his first works. When insects were introduced into a double-choice arena with a human-scented side and a neutral side, they moved to the stimulus side almost immediately after crossing through the nonstimulus section. Similar experimental designs also found that the attractiveness of human odor was enhanced by lice odor and their excreta. Mumcuoglu et al. (1986) validated and upgraded research on the attraction of *Pediculus humanus humanus* to its excretory products, identifying the chemical compounds present in the louse feces such as hypoxanthine, hemoglobin, uric acid, xanthine, and ammonium salts. Attraction in head lice was recently analyzed by Ortega-Insaurralde et al. (2016) and Galassi et al. (2018). In the first report, the authors showed for the first time that adult lice responded to human scalp substances. The authors found that scalp compounds induced head lice to decrease average locomotor activity and to remain arrested on the treated paper. They also observed behavior associated with the search for blood on the skin surface, such as stillness, body positioning perpendicular to the surface, and head disposition near the surface. Later, Galassi et al. (2018) showed that lice were specifically attracted to volatiles from human scalp. The volatiles were identified and the attraction towards the main individual compounds (nonanal, sulcatone, geranylacetone, and palmitic acid) was tested. The authors demonstrated that nonanal activity depends on the concentration of the compound, as it is repellent at high concentrations and an attractant at low concentrations. More recently, these authors showed that lice were highly attracted to human odor volatile compounds, but showed no preference for volatiles from the head compared to volatiles from other parts of the body. In contrast, when lice were exposed to whole human extracts from different parts of the body, they showed a preferential response to head odor compared to foot or forearm. These results suggest that head lice can, at short distances within the host, be oriented towards the scalp environment (Galassi et al. 2019).

Mixtures of odors are used in nature in order to increase the certainty that an odor source belongs to an expected host (Lehane 2005). As reported for other species, although individual compounds can trigger behavior, insects generally respond better to mixtures than to isolated compounds (Riffell et al. 2009a). For ticks, Ferreira Borges et al. (2015) showed that the beagle breed of domestic dogs produces natural repellents against the *Rhipicephalus sanguineus* tick. This work compared the chemical profiles of a tick-attractive breed (Cocker Spaniels) with those from nonattractive breeds, finding specific compounds in Beagles that produce repellency towards ticks in an olfactometer test. Beagle's skin compounds produced an increased repellency when they were tested as a blend. Furthermore, for the *Aedes aegypti* (L.) mosquito, a synergistic attraction for binary mixtures of L-lactic acid plus acetone, dichloromethane, or dimethyl disulfide was reported (Bernier et al. 2003).

In this study, we evaluated the response of head lice towards mixtures of head scalp chemicals identified both from volatile emanations and from solvent extracts. These responses were compared to the responses towards the isolated compounds.

Materials and Methods

Head Lice

Adult head lice were obtained from infested children 6–12 yr old of elementary schools from Buenos Aires, by combing with a fine-toothed metal comb (Assistance, Buenos Aires). Head lice were stored into plastic boxes and transported to the laboratory. Lice were classified in the laboratory and those with damaged legs or antennae were discarded. Male and female adult head lice were used in the experiments and results were analyzed together considering that both sexes have the same diet.

Lice were kept in an environmental chamber at 18°C and 70% RH (Picollo et al. 1998) until they were used. They were also fasted at least for 1 h before bioassays, to avoid differences in fasting state between experimental trials. All lice used in bioassays were tested within 3 h after collection and protected from sunlight and heat. The protocol for louse collection was approved by the ad-hoc committee of the Centro de Investigaciones de Plagas e Insecticidas, UNIDEF, Buenos Aires, and archived in our institution (#BA20061995ARG, June 1995) (Picollo et al. 1998).

Chemicals

Nonanal (95%), sulcatone (99%), geranylacetone (>97%), palmitic acid (99%), palmitoleic acid (99%), tetradecanoic acid (>98%), oleic acid(>99%), and squalene (>98%) were purchased from Sigma-Aldrich (St Louis, MO). Acetone analytical grade (99.8%, Merck Darmstadt, Germany) was used as solvent in bioassays.

Study 1: Response of Head Lice Towards Mixtures of Head Scalp Volatiles

The evaluation of the behavioral response of lice towards nonanal, sulcatone, and geranylacetone and their mixtures was carried out in an adapted T-tube olfactometer (Galassi et al. 2018). The olfactometer was set in a closed chamber at low-intensity light (21 lux), 50% RH, and 30°C (Ortega-Insaurralde et al. 2015). A strip of filter paper was placed inside the olfactometer (220 x 3 mm) to allow louse crawl.

The effect was evaluated by placing a treated paper into a 2 ml vial on one arm of the olfactometer and a control paper (acetone) at the other arm. The air flow through the olfactometer, measured with an anemometer, was pumped at 5 cm/s. In each test, a head louse was gently introduced into the olfactometer through the central opening and allowed to walk freely for 30 s for acclimatization plus 150 s for evaluation. At the end of the experiment, the arm selected by the louse was recorded and a binary value was assigned (*one* means selection of treated side and *zero* untreated side). After each bioassay, the head louse was discarded and the device was cleaned with ethanol. To discard a bias towards one side of the olfactometer, the position of control and treated papers was alternated. Twenty replicates (an individual louse was considered a replicate) of each compound and twenty replicates of the two mixtures of components were tested in two concentrations (*n:* 320 insects). For the control trials, untreated filter papers were placed at both arms of the olfactometer. This same experimental methodology was implemented in Galassi et al. 2018. Every head louse was used only once in this bioassay, then it was discarded.

In Galassi et al 2018, the authors observed an increasing attraction to head lice was found by nonanal, sulcatone, and geranylacetone at low concentrations, so dilutions of each compound in acetone were prepared at individual concentrations of 0.005 mg/ml and 0.015 mg/ml. Ternary mixtures of the individual compounds were tested. The final concentrations were reached by adding concentrations of the individual components (ternary mixture of 0.005 mg/ml= 0.00167 mg/ml nonanal + 0.00167 mg/ml sulcatone + 0.00167 mg/ml geranylacetone; mixture of 0.015 = 0.005 mg/ml nonanal + 0.005 mg/ml sulcatone + 0.005 mg/ml geranylacetone). Ten microliters of each solution were applied onto a Whatman #1 filter paper square (1.5 cm side) to get final masses of 0.05 and 0.15 micrograms of sample on the filter papers.

Statistical Analysis

The preference of the louse towards the treated or untreated side of the olfactometer was registered as a binary data and analyzed using a GLM with logit link function and binomial structure with n:1 (Bernoulli distribution) (Zuur et al. 2009). Data analyses and functions were conducted by packages from RStudio (R Core Team 2016). The trial design assured the assumptions of randomness and independent data, as treated and untreated filter paper squares were randomly alternately assigned to left or right area. Each insect was tested only one time.

A Hosmer-Lemeshow test checked the goodness of fit to the model (Paul et al. 2012, Surjanovic and Loughin 2021) and drop1 function was used to retain the significant factors of the model (Hodgkiss et al. 2018). RStudio's package ResourceSelection (Lele et al. 2019) was used in the statistical analysis.

Study 2: Chemical Identification of Human Scalp Solvent Extract

Collection of Human Scalp Extract

The collection of human scalp extract was carried out on a young adult male volunteer whose head scalp's attraction to lice was previously demonstrated. Two days prior to the collection, he showered with a fragrance-free shampoo (Biferdil, Buenos Aires) to lessen the impact of exogenous sources of chemical compounds.

For collection, 2 ml of a methanol-acetone mixture (1:1) was applied with Pasteur pipette on a scalp surface limited by a 2.5 cm glass tube. This procedure was repeated 3 times (2 ml each time) to ensure maximum extraction of scalp compounds.

The resulting extract was centrifuged at 4.000 RPM to separate the particulate components, concentrated to 50 microliters by evaporation with nitrogen, and kept at –78°C until analysis.

Chemical Identification

The extract was analyzed on a Shimadzu QP 2010 Ultra Gas Chromatograph Mass Spectrometer, equipped with a DB-WAX capillary column (30 m, 0.25 µm, 0.25 µm, Agilent, Santa Clara, CA), with helium as carrier gas (column flow 1.4 ml/min). The GC oven temperature was programmed from 60°C (4 min hold), followed by a ramp of 6°C/min to 220°C and held iso thermic for 10 min, and finally a ramp of 1°C/min to 245°C and held isothermic for 10 min. The injector was kept at 240°C. The detector was operated at 70 eV, scanning from 40 m/z to 350 m/z and 245°C interface temperature.

The chemical identification was carried out using standard reference samples obtained from Sigma-Aldrich (St Louis, MO), by comparing its retention index (RI) with literature data and/or by the comparison and analysis of the mass spectrum (MS) against the Wiley Mass Spectra Library (McLafferty 2005). The compounds identified with relative area major to 1.5 were selected to be evaluated individually and in mixture in the bioassays described in Study 3.

Study 3: Behavioral Response to Human Scalp Solvent Extract

The evaluation of the response of head lice to components of human solvent extract was performed in an experimental arena consisting of a 5.5 cm circular filter paper (Whatman #1, GE Healthcare, Little Chalfont, UK) divided into two symmetrical areas. The arena was enclosed with a 5 cm circular glass ring to prevent insect escapes and kept at 21 lux light and 30°C temperature (Ortega-Insaurralde et al. 2015).

An 1.5 cm filter paper square impregnated with the test sample was placed on one half of the arena and an untreated filter paper on the other. One insect was gently placed in the center of the arena and allowed to walk freely for 180 s. The insect movements were observed by an infrared camera (KIR-J639CE20, Sony, China) and recorded by a digital video (DVR5104HE, Dajua Technology Co Ltd, Hangzhou, China). The control arena consisted of the divided circular filter paper with one untreated filter paper square on each side (Ortega-Insaurralde et al. 2016).

In order to estimate the natural content of these compounds in the scalp, a semi-quantitative gravimetric determination was performed. A weighted 1.5 x 1.5 cm square filter paper was rubbed against the scalp for 30 s and weighed pre and after in an analytical scale (Shimadzu, model AUY 220). Five replicates were taken and the average scalp sample was calculated. With the result of the total mass obtained and the relative areas obtained for each component by GC-MS, the amount present of each component of head scalp was estimated.

Major individual components identified in *Study 2* were tested in the bioassay. The solutions tested were 1.06 mg/ml palmitic acid, 2.12 mg/ml palmitoleic acid, 0.53 mg/ml tetradecanoic acid, 2.12 mg/ml oleic acid, and 42.4 mg/ml squalene. As these concentrations were the ones present in the natural extract according to our gravimetric determination, a mixture of them was evaluated (1.06 mg/ml of palmitic acid + 2.12 mg/ml of palmitoleic acid + 0.53 mg/ml of tetradecanoic acid + 2.12 mg/ml of oleic acid + 42.4 mg/ml of squalene). The five-component blend was also evaluated in a 1/5 dilution (0.212 mg/ml of palmitic acid + 0.424 mg/ml of palmitoleic acid + 0.106 mg/ml of tetradecanoic acid + 0.424 mg/ml of oleic acid + and 8.48 mg/ml of squalene). Ten microliters of each sample were applied on a filter paper to obtain their corresponding masses. Twenty replicates of each compound and blend were performed (*n*: 280 insects). Every head louse was used only once in this bioassay, then it was discarded.

Statistical Analysis

The permanence of head lice on each side of the arena was analyzed by one-way ANOVA. All analyses and functions were conducted by means of packages from RStudio (R Core Team 2016). Normality and heteroscedasticity were ascertained through Shapiro-Wilk and Levene tests. Outliers were detected and replaced by other assay's results (Osborne and Overbay 2004). In case of assumptions that were not accepted, Varianze model functions were tested: VarIdent, VarPower, and VarExp (Zuur et al. 2009). Initial model and Varianze modeled candidates were compared by AIC (Wagenmakers and Farrell 2004), VarPower was the most appropriate to model variance and fit normality, tested by Levene test and Q-Q plot respectively. A Tuckey "a posteriori" classification of model was carried out to establish the differential set of data. RStudio's packages nlme (Pinheiro et al. 2016), MASS (Venables et al. 2002), car (Fox and Weisberg 2011), rapportools (Blagotic and Daróczi 2014), and emmeans (Lenth 2021) were used in statistical analysis.

Results

Study 1: Response of Head Lice Towards Mixtures of Head Scalp Volatiles

In the olfactometer tests, the head lice showed attraction towards the ternary mixture compared to its control in the concentrations of 0.015 mg/ml ($P = 0.0577$) and 0.005 mg/ml ($P = 0.0528$). The drop1 test presented a significant result in concentration 0.005mg/ml ($P = 0.04$). This effect of attraction was not observed for the individual components (Fig. 1).

Study 2: Chemical Identification of Solvent Extract

The components identified in the scalp extract by GC-MS are shown in Table 1. In total, 24 compounds were identified, the most abundant being squalene (78.9%), palmitoleic acid (4.42%), palmitic acid (2.9%), tetradecanoic acid (1.95%), and oleic acid (4.52%).

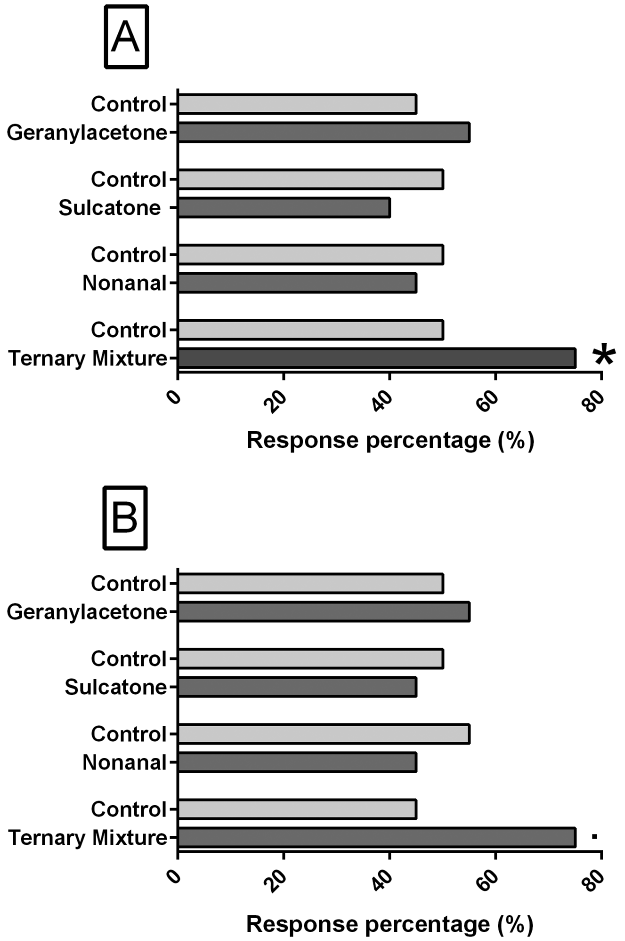

Fig. 1. Response of head lice towards major head scalp volatiles and their ternary mixture. A: concentration 0.005 mg/ml. *: Statistically significant in drop1 test ($P = 0.04$). B: concentration 0.015 mg/ml.: ($P = 0.0577$).

In smaller proportions, the following chemicals were identified: undecane, 1,2-dimethylbenzene, 1,2,4-trimethylbenzene, 1,2-propanediol, 3,7,11,15-tetramethyl-2-hexadecene, 1-undecanol, ethyl myristate, ethyl pentadecanoate, 1-dodecanol, hexadecanoic acid methyl ester, 1-tetradecanol, hexadecanoic acid ethyl ester, glycerol, octadecanoic acid ethyl ester, ethyl oleate, tridecanoic acid, 1-octadecanol acid, pentadecanoic acid, and heptadecanoic acid. For bioassays, compounds with a relative area greater than 1.5% were selected.

Study 3: Behavioral Response to Human Scalp Solvent Extract

In the bioassays carried out in the experimental circular arena, lice were attracted to the quintuple blend compared to their controls (*P*-value 0.0495). The individual compounds (palmitic acid, palmitoleic, tetradecanoic, oleic acids, and squalene) and the 1/5 diluted mixture did not show a differential response compared to the control (Fig. 2).

Discussion

This work identified the human scalp solvent extract composition and evaluated how head lice are attracted to isolated compounds and blends. We also tested the attraction of volatile head scalp components previously identified in our lab (Galassi 2018).

Concerning the role of volatile components from head scalp in the attraction of head lice, the main components that were individually evaluated did not show an attraction effect towards head lice. However, the mixture of the three major volatiles (nonanal, sulcatone, geranylacetone) of the human scalp showed an attractive response towards head lice.

GC-MS was used to identify the compounds present in human scalp solvent extract. The main compounds were squalene and to a lesser extent four acids: palmitoleic, palmitic, tetradecanoic, and oleic. These compounds are characteristic of the human scalp and are secreted by the sebaceous glands (Nicolaides 1974). The secretions of the sebaceous glands are rich in lipid materials such as cholesterol, cholesterol esters, long-chain fatty acids, squalene, and triglycerides. These lipids provide a substrate for the growth and metabolism of skin microorganisms. Furthermore, the presence of these compounds was well established in different subjects (Gallagher et al. 2008). However, the amount of these compounds present in the skin depends on the sex, age and ethnicity of the person (Shetage et al. 2014).

These results are consistent with previous studies in other species where blends were more attractive than isolated compounds. For example, the grapevine moth, *Lobesia botrana*, shows differential attraction to certain blends compared with the response to individual compounds (Tasin et al. 2007). Similar results were found in the hematophagous insect, *Triatoma infestans*, when the response to carbon dioxide and other odors of vertebrate origin were analyzed in a locomotion compensator. The insects did not show an oriented response to L-lactic or ambient carbon dioxide concentrations, but they showed a marked synergism when L-lactic acid was combined with a sub-threshold concentration of carbon dioxide (Barroso and Lazzari 2004).

Smith et al. (1970) studied L-lactic as a factor in attraction to *Ae. aegypti* at different concentrations. They also tested attraction in the presence of carbon dioxide, proving that attraction is increased when both chemicals were present in a mixture. Another study on *Ae. aegypti* reported the attraction response to separate components from human skin, and three binary blends (Bernier et al. 2003). The blends composed of L-lactic plus either acetone, dichloromethane, or dimethyl disulfide, synergistically attracted laboratory-reared female mosquitoes. The authors postulated that at least one of these synergistic blends (L-lactic acid and acetone) produced the insect attraction behavior similar to L-lactic acid and carbon dioxide.

In the present work, the biological evaluation of the five main components present in head scalp extract towards head lice showed that they were individually not attractant. However, their mixture in a proportion similar to the natural proportion of the human volunteer showed a higher response when compared to controls and to individual components. Several works support the fact that single components from an active extract have low or no activity when they are individually evaluated, and that they increase their bioeffect when combined in mixtures of different compositions.

Braz-Louly et al. (2010) studied the response of the brown dog tick, *R. sanguineus,* to extract from cocker spaniel and beagle dogs, and found that the ticks were attracted by cocker spaniel extracts and were repelled by compounds from beagles, i.e. 2-hexanone, benzaldehyde, nonane, decane, and undecane. In bioassays, 2-hexanone and Benzaldehyde repelled ticks but when 2-hexanone and benzaldehyde were combined, the repellency increased such that it was comparable or better than N,N-diethyl-3-methylbenzamide (DEET).

Later, Ferreira Borges et al (2015), identified nonhost semiochemicals for the brown dog tick, *R. sanguineus,* in the tick-resistant beagle dog compared with the tick-susceptible cocker

Table 1. Compounds from human scalp detected by GC-MS analysis

#	RET time	Area (%)	Compound	Literature RI	Relative RI	Match (%)	Std
1	3.9	0,04	undecane	1100	1102	94	b
2	4.9	0,03	benzene, 1,4-dimethyl	1377	1227	95	a
3	8.2	0,04	benzene, 1,2,4-trimethyl	1285	1281	92	b
4	15.4	0,23	1,2-propanediol	1853	1847	92	a
5	29.7	0,04	3,7,11,15-tetramethyl-2-hexadecene	1926	1934	91	b
6	22.4	0,1	1-undecanol	2041	2049	93	a
7	23.7	0,25	ethyl myristate	2043	2036	92	b
8	25.3	0,27	ethyl pentadecanoate	2172	2170	91	b
9	25.7	0,15	1-dodecanol	2189	2180	96	a
10	26.3	0,06	hexadecanoic acid, methyl ester	2109	2112	93	b
11	28.7	0,3	1-tetradecanol	2175	2154	97	a
12	26.9	0,7	hexadecanoic acid, ethyl ester	2243	2249	93	b
13	27.7	1,47	glycerol	2322	2317	92	a
14	29.9	0,07	octadecanoic acid, ethyl ester	2442	2432	92	b
15	30.1	0,19	ethyl oleate	2470	2474	93	a
16	34.0	1,24	tridecanoic acid	2570	2571	93	a
17	31.7	0,42	1-octadecanol	2585	2586	95	a
18	35.1	1,95	tetradecanoic acid	2724	2725	94	a
19	36.5	4,42	palmitoleic acid	2960	2663	93	a
20	36.8	0,56	pentadecanoic acid	2822	2832	92	a
21	39.1	2,9	palmitic acid	2931	2924	92	a
22	45.2	78,9	squalene	2914	2894	94	a
23	47.2	0,59	heptadecanoic acid	3027	3024	93	a
24	38.7	4,52	oleic acid	3154	3147	92	a

"a" ID confirmed also by comparison with synthetic standard, "b" synthetic standard, "IR" retention index

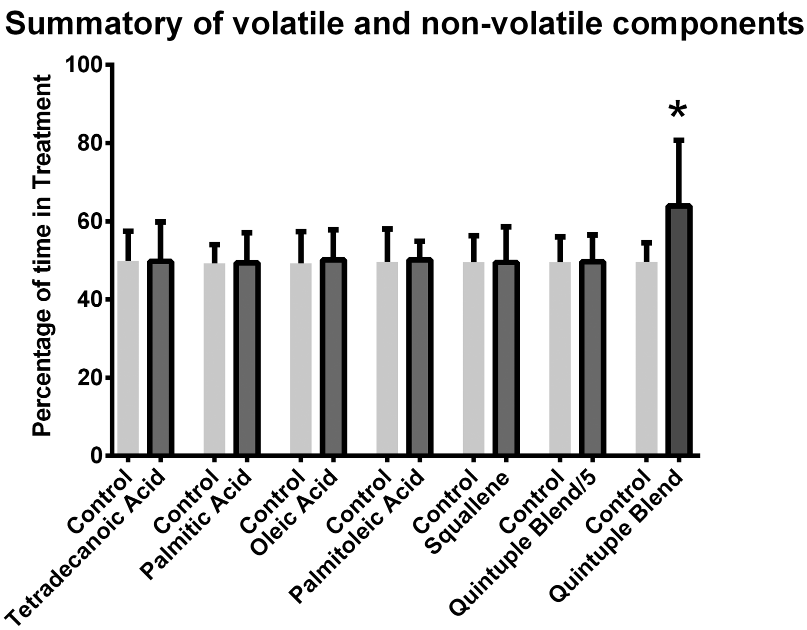

Fig. 2. Percentage of time spent by head lice on treatment side versus control. Individual compounds were tested at natural concentration. Quintuple Blend is the mixture of the five compounds at natural concentration. Quintuple Blend/5 is the dilution of quintuple blend by a 1/5 factor. Mean and standard deviations bars are plotted for each treatment and control. *: significant difference ($P = 0.0495$).

spaniel dog. They evaluated the attractive components of the host odors towards the ticks by exposing them to single chemicals and to their mixtures. The beagle odor extracts contained almost three times as many chemical compounds as cocker spaniel extracts. Several nonhost compounds were identified, i.e. 2-hexanona, benzaldehyde, nonane, and undecane. None of the components were significantly repellent when evaluated at different concentrations or times. But when benzaldehyde and 2-hexanone were combined, an increase in the repellency rate was registered, and the activity was similar to N,N-diethyl-3-methylbenzamide. This suggested that nonhost semiochemicals mediated the avoidance of the beagle dog breed by *R. sanguineus*.

Concerning the role of carbon dioxide and heat in head louse behavior, to our knowledge, this has not been assessed. Unlike mosquitoes and kissing bugs, the head louse is not an active seeker, so they do not need long-distance factors for host-seeking.

This work found that blends from human scalp compounds are attractive to human head lice at certain concentrations. These results suggest that the mixture of compounds from the human scalp can orient the insects to their habitat: the human head. This work contributes to our understanding of the attractivity of some individuals and not others for lice, and could explain the difference in attractivity between young children and adults, and even among susceptible and nonsusceptible children.

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