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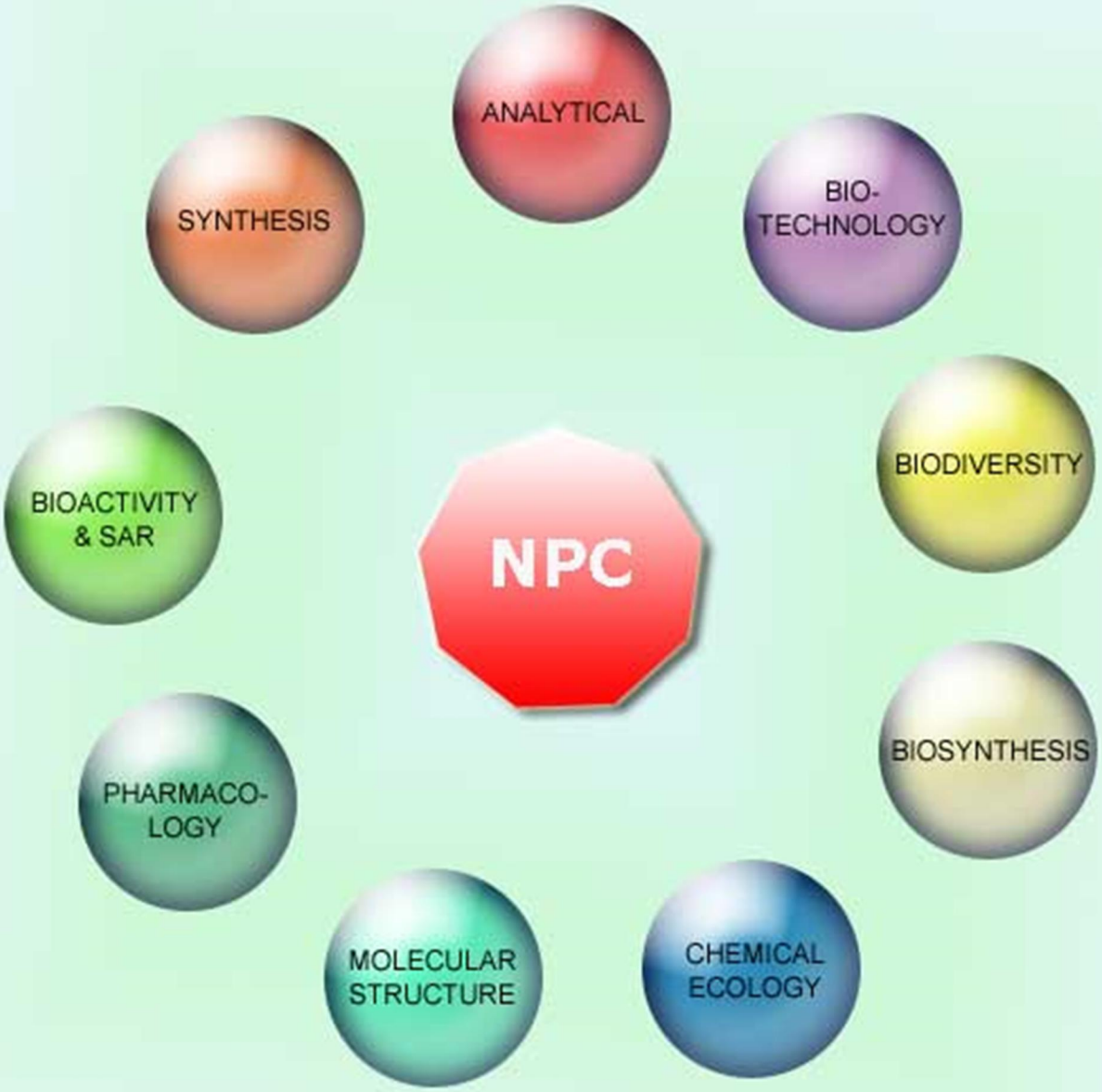

This Issue is Dedicated to
Professor Josep Coll Toledano
On the Occasion of his 70th Birthday

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Cuticular and Internal Chemical Composition of Biting Midges *Culicoides* spp. (Diptera: Ceratopogonidae), Potential Vectors of Viral Diseases

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The chemical profile of the cuticle and internal tissues of four species of *Culicoides* have been studied for the first time by gas chromatography-mass spectrometry. The chemical composition of females of *C. obsoletus* s.l. and *C. lupicaris*, vectors of diverse viral diseases, have been compared with that of other biting midges, such as *C. kibunensis* and *C. fascipennis*, and the non-biting midge *Forcipomyia bipunctata*. A total of 61 compounds belonging to 8 major chemical classes were identified in cuticular and internal tissues in *n*-hexane extracts. The compounds include carboxylic acids (CAs) (C6-C20), with C16:0, C16:1 and C18:1 being dominant, branched hydrocarbons (C29 to C38 mono/di/trimethylalkanes), linear hydrocarbons (C15 to C33, mainly odd chain carbons), terpenes (geranylacetone, geranylgeraniol acetate, squalene, terpenic alcohol), steroids (cholesterol), aldehydes (C9-C10 and even chain C20 to C30), and esters. The chemical profile depends on the species and whether the extracts are external (cuticle) or internal. The contents of linear and branched hydrocarbons and aldehydes was high in cuticular extracts but practically absent in internal tissues, which were, in contrast, rich in CAs, terpenes and steroids. The results are discussed and compared with other *Culicoides* midges and mosquito-related species.

Keywords: Biting midges, *Culicoides obsoletus*, *Culicoides lupicaris*, *Culicoides kibunensis*, *Culicoides fascipennis*, *Forcipomyia bipunctata*, Cuticular composition, Internal tissue composition.

Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are tiny haematophagous insects, worldwide known as vectors of viruses which cause diseases of major international relevance. These viruses comprise the Bluetongue virus (BTV) [1], the African-horse sickness virus (AHSV) [2], the Epizootic haemorrhagic disease virus (EHDV) [3] and the Schmallenberg virus (SBV) [4], among others. Knowledge of the taxonomy, epidemiology and bioecology of these insects has received great interest in the last years after several BTV outbreaks in the Mediterranean regions and other countries of North and Central Europe [5].

The cuticle of insects plays an important role in numerous biochemical, physiological and semiochemical processes [6]. Cuticular compounds may act as inducers of insect resistance to entomopathogens (bacteria, fungi) [7], inhibitors of fungal attachment [6c], and antimicrobial agents [8]. In particular, cuticular hydrocarbons (HCs) were proposed as a taxonomic tool for identification and recognition of insects of medical interest [9], and as inter and intraspecific communication agents in insects [6a,6c,10]. However, the role of cuticular compounds in *Culicoides* spp. remains unknown. In *C. melleus,* a mixture of methyl substituted *n*-alkanes of C22 and C23 from the female cuticle could be a contact pheromone stimulating males for copulation [11]. Later, the same authors reported the first cuticular HCs in *C. melleus* and *C. variipennis* and their possible role as putative sex pheromones [12]. In *C. impunctatus,* females appear to produce an aggregation or recruitment pheromone to attract individuals of the same sex [13], and in *C. nubeculosus* females produce *n*-heptadecane as a sex pheromone to attract males for mating [14]. Other cuticular compounds of five species of *Culicoides* from non-Palearctic regions were identified by GC as mixtures of fatty acids, but no biological significance was reported [15]. We present herein, for the first time, the chemical profile of the cuticle and internal tissues of the biting midges *C. obsoletus* s.l. and *C. lupicaris,* the two most abundant species in Northern Spain and major potential vectors of BTV in North Central Europe, in comparison with *C. kibunensis* and *C. fascipennis*, and the non-biting midge *Forcipomyia bipunctata.* The results are compared with the cuticular composition of other insects, particularly blood-feeding mosquitoes. Some of the identified compounds have proved to play a role in the chemical communication of these insects.

The cuticular and internal tissue profiles of the biting midges *Culicoides* spp. and the non-biting midge *F. bipunctata* are complex mixtures of diverse families of chemicals, being predominantly linear and methyl-branched HCs, carboxylic acids (CAs), terpenes, steroids, aldehydes and esters (Table 1, Figure 1). Also, there is a wide variability between the cuticular and internal tissue extracts within the same species.

A total of 61 different compounds have been identified (Table 1). The compounds include 27 HCs (14 *n*-alkanes, 3 methylalkanes, 7 dimethylalkanes, 2 trimethylalkanes, and one alkene), 14 CAs (8 saturated and 6 unsaturated), 9 esters (7 saturated, 2 unsaturated), 8 aldehydes (saturated), 4 terpenes, one steroid, one alcohol and one lactone (Table 1). The relative amount of the different chemical families is shown in Figure 2. The cuticle of *C. obsoletus* s.l. and of

Table 1: List of compounds identified in cuticular and internal extracts of *Culicoides* spp. and *Forcipomyia bipunctata* females.

				C. obsoletus [a]		*C. lupicaris* [a]		*C. kibunensis* [a]		*C. fascipennis* [a]		*F. bipunctata* [a]	
N	**ID**	**MW**	**Compound**	**CU**	**IN**	**CU**	**IN**	**CU**	**IN**	**CU**	**IN**	**CU**	**IN**
		HYDROCARBONS											
1	S/CI/L	212	*n*-Pentadecane C15	-	-	-	-	-	-	-	-	+	t
2	S/CI/L	238	*n*-Heptadecene [b]	-	-	-	-	-	-	-	-	+	-
3	S/CI/L	240	*n*-Heptadecane C17	+++	+	++	+	++	+	+	+	++	+
4	S/CI/L	254	*n*-Octadecane C18	-	-	-	t	-	-	-	-	-	-
5	S/CI/L	268	*n*-Nonadecane C19	++	-	+	+	+	+	+	-	+	t
6	S/CI/L	296	*n*-Henicosane C21	t	t	t	t	-	-	+	-	-	-
7	S/CI/L	324	*n*-Tricosane C23	++	t	++	+	++	-	+++	-	+	-
8	S/CI/L	352	*n*-Pentacosane C25	++	++	++	+	++	++	+	++	++	t
9	S/CI/L	366	*n*-Hexacosane C26	-	-	+	-	-	-	t	-	t	-
10	S/CI/L	380	*n*-Heptacosane C27	++	++	++	+	++	++	++	++	++	+
11	S/CI/L	394	*n*-Octacosane C28	-	-	+	-	-	-	t	-	-	-
12	S/CI/L	408	*n*-Nonacosane C29	++	++	++	+	++	+++	+	++	+	t
13	S/CI/L	422	11-/13-/15-MeC29 [c]	-	-	-	-	-	-	-	-	++	-
14	S/CI/L	436	11,13-DiMeC29 + 13,15-DiMeC29 [d]	-	-	-	-	-	-	-	-	++	-
15	S/CI/L	436	*n*-Hentriacontane C31	+	-	-	-	-	-	-	-	-	-
16	S/CI/L	464	*n*-Tritriacontane C33	++	-	-	-	t	-	t	-	-	-
17	L	492	15,19-DiMeC33	-	-	-	-	++	-	-	-	-	-
18	L	506	15,18-DiMeC34	-	-	-	-	-	-	++	-	-	-
19	CI/L	506	13-/15-/17-MeC35 [c]	++++	t	+	-	++	-	++++	-	+	-
20	CI/L	520	15,19-DiMeC35	-	-	-	-	-	+	-	-	-	-
21	CI/L	520	11,14-DiMeC35 + 13,16-DiMeC35	+++++	t	+++	t	++++	-	++	-	+	-
22	CI/L	534	11,15,19-TriMeC35	t	t	++	t	-	-	++	-	-	-
23	CI/L	534	13,17,21-TriMeC35	-	-	-	-	+++	-	-	-	-	-
24	CI/L	548	11-/13-MeC38 [c, d]	-	-	+	-	++	-	+	-	-	-
25	L	548	MeC38 mixture [d]	-	-	+	-	++	-	-	-	-	-
		CARBOXYLIC ACIDS											
26	CI/L	116	Hexanoic acid	-	-	-	-	-	-	-	+	-	-
27	L	144	2-Ethyl hexanoic acid	-	t	-	t	-	t	t	+	-	-
28	CI/L	144	Octanoic acid	-	t	-	+	-	t	-	+	t	t
29	CI/L	158	Nonanoic acid	+	+	+	+	+	+	+	++	+	t
30	CI/L	172	Decanoic acid	-	t	-	t	-	t	-	-	-	-
31	L	228	Tetradecanoic acid	-	-	t	+	++	++	-	+	+	-
32	CI/L	254	Hexadecenoic acid [b]	+++	++++	++++	++++	++	++++	++++	++++	+++++	++
33	S/CI/L	256	Hexadecanoic acid	++	+++	+++	++++	++	+++	++++	++++	+++	++
34	L	268	Heptadecenoic acid [b]	t	-	t	-	-	-	-	-	-	-
35	S/CI/L	280/282	Linoleic + oleic acid	+++	+++++	++++	+++++	++	+++++	+++	+++++	+++++	++++
36	CI/L	284	Octadecanoic acid	-	+	+	+++	+	+	+	++	+	+
37	CI/L	306	Arachidonic acid + dihydroarachidonic acid [b, d]	+	-	++	-	t	-	++	-	++	-
		ESTERS											
38	L	242	iso-Propyl dodecanoate	-	-	-	t	-	-	-	+	-	-
39	L	270	iso-Propyl tetradecanoate	+	++	t	++	t	++	t	++	-	-
40	L	268	Methyl 9-hexadecenoate	-	-	-	-	-	-	-	-	-	+
41	L	284	Ethyl hexadecanoate	-	++	-	t	-	-	-	+	-	-
42	L	298	iso-Propyl hexadecanoate	+	++	+	++	-	++	t	+++	-	-
43	L	312	Isooctyl dodecanoate [d]	+	t	-	-	-	-	-	t	-	-
44	L	310	Ethyl octadecenoate [b]	-	++	-	-	-	-	-	-	-	-
45	L	312	*n*-Octadecyl acetate	-	-	-	t	-	-	-	++	-	-
46	L	340	*n*-Eicosanyl acetate	-	-	-	t	-	++	-	++	-	-
		ALDEHYDES											
47	CI/L	142	Nonanal	t	-	-	t	t	+	+	t	-	-
48	CI/L	156	Decanal	t	-	t	t	t	t	+	+	-	-
49	CI/L	296	Icosanal	-	-	+	t	-	-	-	-	-	-
50	CI/L	324	Docosanal	-	-	++	-	-	-	-	-	-	-
51	S/CI/L	352	Tetracosanal	t	-	++	-	-	-	-	-	-	-
52	CI/L	380	Hexacosanal	-	-	+	-	-	-	-	-	-	-
53	CI/L	408	Octacosanal	-	-	++	-	t	-	-	-	-	-
54	CI/L	436	Triacontanal	-	t	+	-	t	-	-	-	-	-
		TERPENES											
55	L	194	Geranylacetone	t	t	-	t	t	t	t	+	t	t
56	CI/L	332	Geranylgeraniol acetate [d]	-	-	-	-	+	-	-	t	+	++++
57	S/CI/L	410	Squalene	++	+++++	+	+	+	++	t	++++	-	t
58	L	--	Terpenic alcohol [d]	-	-	-	++	-	+	-	+++	++	++++
		STEROIDS											
59	S/CI/L	386	Cholesterol	+++	+++++	+	++++	+++	+++++	+++	++++	+	-
		ALCOHOLS											
60	S/L	242	*n*-Hexadecanol	+	+	+	-	-	-	-	-	-	-
		LACTONES											
61	CI/L	178	Isocoumarin	-	-	-	-	-	-	-	-	++	++
		UNKNOWN COMPOUNDS											
62	L	422	Unknown 1	-	-	++	-	-	-	++	-	+	-
63	L	--	Unknown 2	-	-	-	-	-	-	-	-	-	+++++

[a]Symbols represent the percentage of each compound relative to the total abundance: t (trace): ≤0.1%; +: 0.1-1%; ++: 1-5%; +++: 5-10%; ++++: 10-20%; +++++: ≥ 20%. CU: Cuticular; IN: Internal; N: Peak number; ID: Identifying source; MW: Molecular weight; S: Standard; CI: Chemical ionization; L: Literature. [b]Position of the unsaturation was not determined. [c]A dash followed by a forward slash (-/) between numbers refers to mixture of isomers. [d]Tentatively identified.

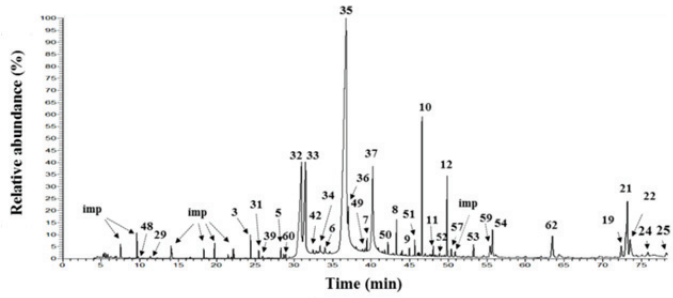

Figure 1: Chromatogram of the cuticular extract of *Culicoides lupicaris* with compounds cited in table 1. Imp: impurity.

C. kibunensis mostly contains branched and linear HCs, accounting for 66% and 74% of the total. In contrast, in *C. lupicaris*, *C. fascipennis* and particularly in *F. bipunctata*, the cuticular extracts contain CAs as the major class of compounds. With regard to the internal tissue extracts, CAs are the most dominant compounds in *C. lupicaris, C. kibunensis,* and *C. fascipennis*, but not in *C. obsoletus* s.l. in which terpenes/steroids are the most abundant (Figure 2). In the non-biting midge *F. bipunctata* the major compounds are geranylgeraniol acetate, oleic and linoleic acids, a terpenic alcohol and an unknown compound (unknown 2 in Table 1).

Composition of cuticular and internal extracts. Hydrocarbons: The main chemical constituents in both types of extracts were odd straight-chain HCs from C17 to C29 (Table 1). C27 was the most abundant in the cuticular extracts of all biting midges, followed by C17, C23 and C29, which was similar to C25. In internal tissues the predominant HCs followed the order C29>C27≈C25 with C23 being almost absent in all *Culicoides* spp. In *F. bipunctata* only the cuticle contained moderate amounts of C17, C27 and C25. In all insects, C15 appeared to be absent in odd straight chain HCs of either extract except in very small amounts in the latter species. Linear HCs of even chain (C12-C28) were found only in trace amounts or absent in many samples of all studied species. Interestingly, monomethyl (11-/13-/15-MeC29; 13-/15-/17-MeC35; 11-/13-MeC38), dimethyl (11,13-diMeC29; 13,15-diMeC29; 15,19-diMeC33; 15,18-diMeC34; 11,14-diMeC35; 13,16-diMeC35) and trimethyl substituted HCs (11,15,19-triMeC35; 13,17,21-triMeC35) were present only in the cuticle of all midges, not in the internal tissue, with 13-/15-/17-MeC35 and a mixture of 11,14-diMeC35 and 13,16-diMeC35 being predominant. The mass spectra of compounds 13-/15-/17-MeC35, 15,19-diMeC33, and 13,17,21-triMeC35 are shown in Figure 3. The mixture of monomethyl derivatives substituted in 13, 15 and 17 positions present the pair diagnostic ions at m/z 196/197, 336/337 for the 13Me derivative; 224/225, 308/309 for the 15Me; and 252/253, 280/281 for the 17Me compound. The 15,19-diMeC33 spectrum shows the diagnostic ions at m/z 224/225 and 295, whereas the trimethyl substituted 13,17,21-triMeC35 presents key ions at m/z 196/197, 365; 224/225, 337; and 267, 295 (Figure 3). Alkenes were not detected in *Culicoides* biting midges, although *Forcipomyia* cuticular washes showed the presence of *n*-heptadecene in very minor amounts (0.1-1%). Our results are consistent with those reported by Linley and Carlson [12] on *C. variipennis* and *C. melleus*. The authors found in the cuticle linear long chain HCs of C21-C33 as the predominant compounds, methyl-branched HCs of C35-C37, primarily at 11, 13 and 17 positions, and dimethyl substituted compounds of similar chain length. However, they did not detect the presence of any trimethylalkane. Other studies carried out on Culicidae of the genera *Anopheles* (f.i. *A. gambiae* and *A. stephensi*) and *Aedes* (f.i. *A. aegypti, A. hendersoni*, and *A. triseriatus*) resulted in the predominant presence of HCs over a wide range of chain length (C10-C47) [9c,9d, 16].

Cuticular HCs have been reported as important constituents of the insect surface in dipterans and known to function as species and sex recognition cues, among them pheromones, allomones and kairomones [17]. In mosquitoes, cuticular HCs may act as pheromones, modulating their mating behavior. For instance, extracts of *Culex quinquefasciatus*, *C. tarsalis*, and *C. pipiens* acted as attractants for conspecific females, and a contact pheromone from the legs of *Culiseta inornata* allowed males to recognize conspecific females [16b]. In *A. gambiae*, the relative amounts of the cuticular HCs *n*-heneicosane and *n*-tricosane, and in *A. aegypti* those of *n*-heptadecane, *n*-pentacosane and *n*-hexacosane were significantly reduced after the female mated [16b]. In midges, a female-produced sex pheromone has been identified, only in *C. nubeculosus*, as *n*-heptadecane [14].

Extracts of volatiles of emerging mixed adults, containing the pheromone with sheep blood odors, significantly increased the number of matings. In *C. melleus*, extraction of freshly-killed males and females yielded a possible contact pheromone, which elicited significant male response at 1 female-equivalent [11]. The active component was not characterized although several methyl substituted alkanes produced significant responses at high concentrations (1 μg).

Carboxylic acids: In all *Culicoides* spp. considered in this work, carboxylic acids (CAs) were more abundant in internal extracts than in the cuticle. The CAs ranged from C6 to C20, the saturated ones corresponded to C6, C8, C9, C10, C14, C16, and C18; the monounsaturated were assigned as C16:1, C17:1 and C18:1 (oleic acid), the diunsaturated as C18:2 (linoleic acid), and a polyunsaturated compound was identified as a mixture of arachidonic (C20:4) and dihydroarachidonic (C20:3) acids (Table 1). In all cases hexadecanoic acid (C16:0), hexadecenoic acid (C16:1), oleic (C18:1) and linoleic acid (C18:2) were predominant. In *F. bipunctata,* in turn, the last three compounds were common in both types of extracts, particularly in the cuticle. The presence of CAs in the cuticle of different species of dipterans has also been noticed [18]. However, in *Culicoides* spp. very few reports have been found. Only in the Asian species *C. obsoletus*, *C. sinanoensis*, *C. nujiangensis*, *C. punctatus* and *C. pulicaris*, Jin-Hua *et al*. identified 10 CAs by gas chromatography [15]. CAs C16:0, C16:1, C18:0, and C18:1 were prominent in most species whereas those of C12:0, C14:0, C18:2, C20:4, C20:0, and C22:0 were present in small-medium amounts. As shown in Table 1, most of these compounds were also found by us, except CAs C20:0 and C22:0, which were undetected in either tissue. Short- and medium-chain CAs from the cuticle have been found to display antimicrobial and fungicidal properties [7b, 19]. For instance, it is noteworthy the toxic effects of CAs C6:0, C7:0, C9:0, C10:0, C18:2 and C18:3 of the fungi *Entomophthora culicis*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* [20], which can be used as biological control agents against larvae and adults of *Culicoides* spp., such as *C. nubeculosus* [21].

Esters: Two isopropyl esters of C14 and C16 were detected, particularly in the internal tissues of the biting midges studied. Ethyl hexadecanoate and ethyl octadecenoate were present almost exclusively in the internal tissues of *C. obsoletus* s.l., *n*-octadecyl acetate in those of *C. fascipennis,* and *n*-eicosanyl acetate in the internal extracts of *C. kibunensis* and *C. fascipennis*. Interestingly, the non-biting midge *F. bipunctata* lacks any ester in either tissue except a very minor amount of methyl 9-hexadecenoate. Fatty esters

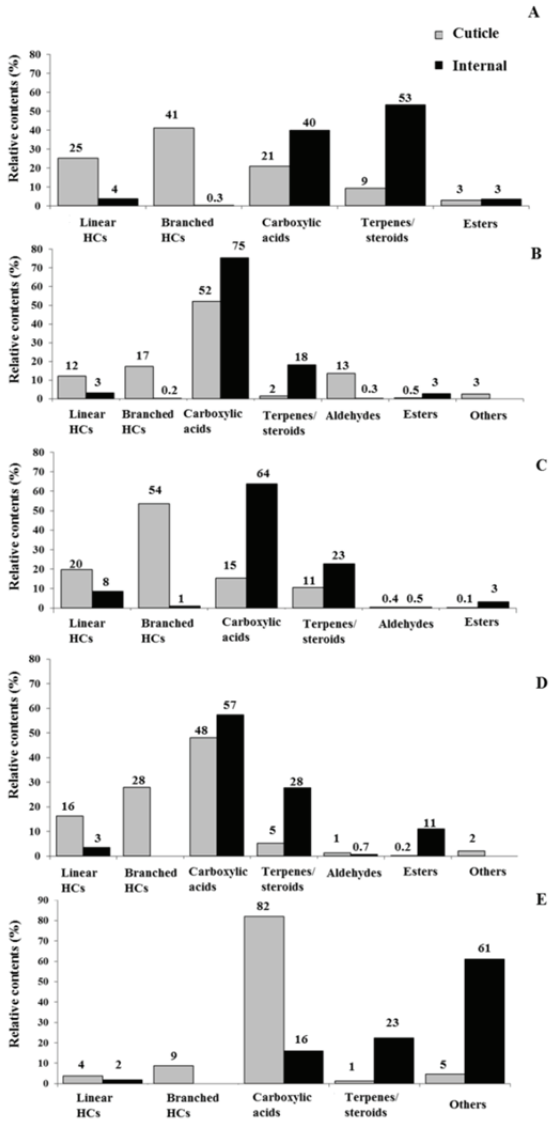

Figure 2: Relative contents of the main chemical classes in cuticular and internal extracts of *C. obsoletus* s.l. (**A**), *C. lupicaris* (**B**), *C. kibunensis* (**C**), *C. fascipennis* (**D**) and *F. bipunctata* (**E**). Others include lactones, alcohols and unknown compounds. For a specific group of chemicals, values <0.5% in both types of extracts have been omitted for clarity.

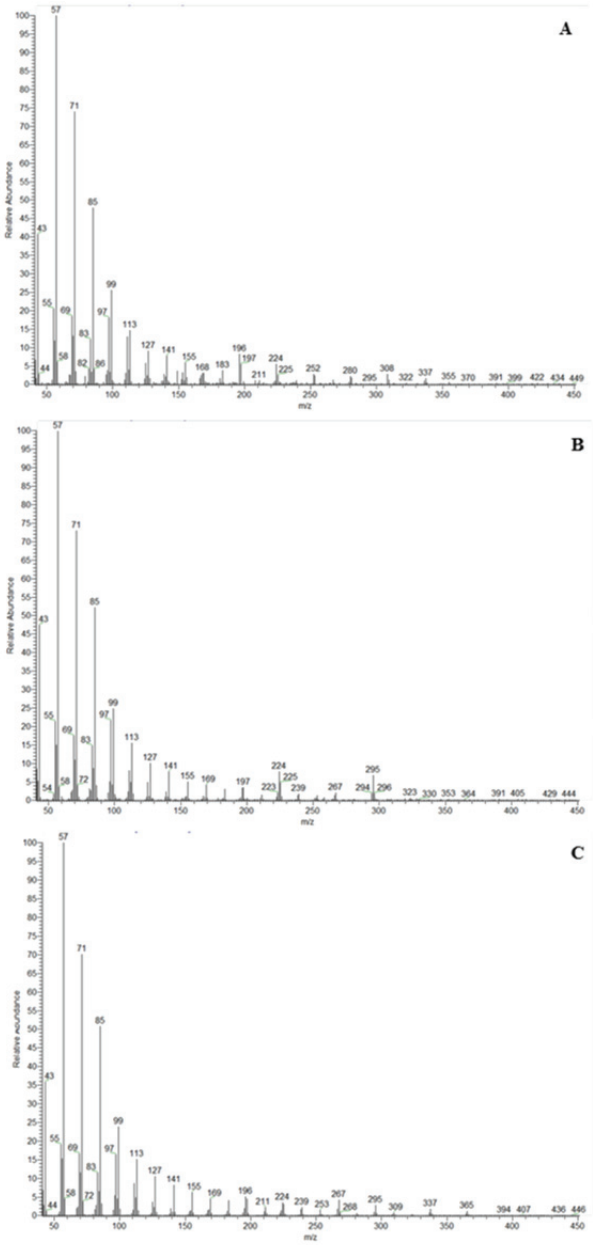

Figure 3: Mass spectra of **A**: compound 13-/15-/17-MeC35 (peak 19); **B**: 15,19-DiMeC33 (peak 17); **C**: 13,17,21-TriMeC35 (peak 23).

are involved in brood recognition and have been demonstrated to act as either pheromones or kairomones in social and non-social insects [22]. However, the role of these esters on *Culicoides* spp. is unknown.

Aldehydes: Aldehydes are not common components of insect cuticular lipids [23]. Thus, in our case, short-chain aldehydes, such as nonanal and decanal, were detected only in very minor amounts in both extracts of *C. kibunensis* and *C. fascipennis*. Long-chain (>C20) even-carbon aldehydes, particularly docosanal (C22), tetracosanal (C24) and octacosanal (C28) were more abundant (1-5%), but only in the cuticle of *C. lupicaris*. In the other *Culicoides* spp. and *F. bipunctata*, aldehydes were practically undetected. Fungistatic properties have been attributed to aldehydes in some insect species [7b, 7c], but not in mosquitoes.

Other compounds: Squalene, the precursor of cholesterol, was present in variable amounts in almost all extracts of *Culicoides* spp. but not in *Forcipomyia*. Cholesterol was the most abundant compound in almost all extracts with, again, the exception of *F. bipunctata*. In general, both compounds were predominant in internal tissues rather than in the cuticle (Figure 2). Cholesterol was also abundant in *A. gambiae* cuticle [9d] and in the non-blood feeder fly, *Lucilia sericata* [20b]. Sterols are also common in assassin bugs [24] and ticks [25].

With regard to alcohols and lactones, to our knowledge no compounds of these classes have been reported previously in any *Culicoides* spp. However, we have detected the presence of *n*-hexadecanol in small amounts (0.1-1%) in both types of extracts of *C. obsoletus* s.l. and in the cuticle of *C. lupicaris,* and a terpenic alcohol in the extracts of some *Culicoides* spp. and, particularly, in the internal tissue of *F. bipunctata.* This latter midge contained also moderate amounts of isocoumarin (1-5%) in both types of tissues. Diverse biological functions of cuticular alcohols and lactones have been reported, but only in Hymenoptera [26] and *Anastrepha* fruit flies [27].

In conclusion, we have provided new data on the chemical characterization of the cuticle and internal extracts of some species of biting midges (*Culicoides* spp.) in comparison with a non-biting midge (*F. bipunctata*) with remarkable qualitative and quantitative differences between groups. Our results provide a first step for further studies on the possible implication of the identified chemicals in the behavior and intraspecific communication of these haematophagous insects.

Experimental

Insect collections: Midges were collected between May 2011 and September 2012 at a sheep farm located in Elguea (42º 55´ 59´´ N and 02º 30´ 51´´ E) in the province of Álava, Basque Country, Northern Spain. The specimens were caught using an ultraviolet CDC light trap model 1212 (J. W. Hock, Gainesville, USA) containing a cubic mesh nest (30x30x30 cm) to minimize insects damage [28].

Insect identification: Insects were maintained in the laboratory in cubic mosquito cages (90x90x90 cm) without any feeding source. Midges were anesthetized under a carbon dioxide stream, manually aspirated and classified to species level under a stereo-microscope (10-40x). Females of the biting midges *C. obsoletus* s.l. *(C. obsoletus/C. scoticus)*, *C. lupicaris, C. fascipennis*, and *C. kibunensis* and the non-biting midge *F. bipunctata* were identified according to their wing pattern [29].

Preparation of extracts: Specimens of different ages were pooled and grouped in 3 different samples (each one considered one replicate) containing 40-60 individuals each. Cuticular extracts were obtained by immersing the insects in glass vials containing 1.5 mL of analytically pure *n*-hexane (SupraSolv, Merck, Darmstadt, Germany), a sufficient volume to cover the entire body of the insects. After 1 h at room temperature (25 ± 3ºC), the extracts were stirred gently by hand (2x) for a short time (approx. 3 s), and the extracts were pipetted out to conical vials with caution to avoid damage of female carcasses. These individuals were then transferred to a glass-mortar (Afora, Fisher Scientific, Madrid, Spain), ground during 15 s in *n*-hexane to extract the internal content. After 5 min, the supernatant was transferred into another new vial. Both extracts (named as cuticular and internal extracts) were stored at -20º C until analysis. Prior to GC-MS analysis, the extracts were evaporated to dryness with a gentle flow of nitrogen, diluted with 10 µL of *n*-hexane, and 2 µL of the new solution was injected into the GC-MS system. Data were analyzed according to their peak areas relative to the total area of all peaks, and the means of the 3 replicates were categorized by symbols according to their abundance: t (trace): ≤0.1%; +: 0.1-1%; ++: 1-5%; +++: 5-10%; ++++: 10-20%; +++++: ≥ 20%. Only peaks that appear at least in 2 of the 3 samples were included in the analysis and compounds present only in trace amounts were excluded from analysis.

Equipment and identification of compounds: Samples were injected in splitless mode into a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadrupole mass spectrometer (ThermoFisher Scientific, Madrid, Spain) in electron impact (EI) mode. Helium was the carrier gas and the analyses were made on an HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm) (Agilent Technologies, Madrid, Spain), and on a SPB-20 column (30 m x 0.25 mm x 0.25 µm) (Supelco, Bellefonte, PA, USA) under the following conditions: injection at 60°C (1 min), and program of 5ºC/min to 180ºC, 2ºC/min to 200ºC, 5ºC/min to 270ºC (hold 20 min), and 5ºC/min to 300ºC (hold 10 min). For chemical ionization (CI) analysis, samples were injected into an Agilent 5973 Network MSD coupled to an Agilent GC 6890 Series using the HP-5MS column cited above, and methane as the reagent gas. The chromatographic conditions used were identical to those used for the EI mass spectra. Compounds were identified by comparison of their MS to those of authentic standards and/or to those already described in the literature [30]. Determination of the branching position of methyl substituted alkanes was based on the fragmentation patterns reported [31] and were supported by their CI-MS when required.

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