Identification of Cattle-Derived Volatiles that Modulate the Behavioral Response of the Biting Midge *Culicoides nubeculosus*

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Abstract

Identification of host-derived volatiles is an important step towards the development of novel surveillance and control tools for *Culicoides* biting midges. In this study, we identified compounds from headspace collections of cattle hair and urine that modulate the behavioral response of *Culicoides nubeculosus,* a research model species with a similar host-range as the vectors of Bluetongue disease and Schmallenberg disease in Europe. Combined gas chromatography and electroantennographic detection (GC-EAD) analysis revealed 23 bioactive compounds, of which 17, together with octanal, were evaluated in a two-choice behavioral assay in the presence of CO_2. Decanal, 2-phenylethanal, 1-octen-3-ol, 2-ethylhexanol, 3-methylindole, phenol, and 3-ethylphenol elicited attraction of host seeking *C. nubeculosus*, whereas heptanal, octanal, nonanal, 3-propylphenol, and 4-propylphenol inhibited the insects' attraction to CO_2, when compared to CO_2 alone. 6-Methyl-5-hepten-2-one, 3-methylphenol, 4-methylphenol, and 4-ethylphenol elicited both attraction and inhibition. The behavioral responses were dependent on the concentration tested. Our results show that cattle-derived odors have the potential to be used for the manipulation of the behavior of *Culicoides* biting midges.
Keywords

Culicoides nubeculosus, Attraction, Behavioral inhibition, Bluetongue, Schmallenberg, Diptera, Ceratopogonidae

Introduction

Culicoides biting midges (*Ceratopogonidae*) are vectors of the Bluetongue virus (Caracappa et al. 2003; Dijkstra et al. 2008; Meiswinkel et al. 2007; Savini et al. 2008; Veronesi et al. 2013b) and the recently isolated Schmallenberg virus (Beer et al. 2013). The introduction and increased incidence of the diseases caused by these viruses have afflicted livestock production significantly in northern Europe (Beer et al.2013; Carpenter et al. 2009). Current vector control management methods, including restrictions on movements of animals, and insecticide treatment of animals, housing, and transport vehicles have had limited effect on the disease dynamics (Carpenter et al. 2008). Surveillance programmes that target biting midges are essential to predicting the spread of these fatal animal diseases. However, current surveillance methods, which rely primarily on light traps to catch biting midges, may not be optimally effective. Such traps may not attract all potential vector species equally, and may underestimate numbers of blood-seeking midges in the vicinity of animal hosts (Carpenter et al. 2008; Gerry et al. 2009; Viennet et al. 2011). Following the successful use of traps baited with vertebrate host volatiles to monitor and control other blood feeding insects (Logan and Birkett 2007; Pickett et al. 2010), similar technology has been trialled

with varying success against biting midges (e.g., Cilek et al. 2003; Gerry et al. 2009; Harrup et al. 2012; Kline et al. 1994; Ritchie et al. 1994). A better understanding of how host-derived chemicals attract, and potentially repel, these insects is essential to developing more effective means of biting midge surveillance and control.

Host preference analysis of Bluetongue and Schmallenberg vector species (Caracappa et al. 2003; Dijkstra et al. 2008; Meiswinkel et al. 2007; Rasmussen et al. 2012; Savini et al. 2008; Veronesi et al. 2013b) show that these insects prefer to feed on cattle, sheep, and horses (Blackwell et al. 1995; Lassen et al. 2011; Pettersson et al. 2012). Field experiments also show that biting midges are differentially attracted to vertebrate host odors when combined with carbon dioxide (CO_2) (Mands et al. 2004), a key kairomone cue for haematophagous insects (Logan and Birkett 2007). Moreover, several generic, vertebrate host-derived volatiles elicit a behavioral response in biting midges, e.g., 1-octen-3-ol and phenolic compounds (Logan and Birkett 2007). 1-Octen-3-ol is one of the best studied host volatiles for haematophagous insects, and attracts tsetse flies (Hall et al. 1984), mosquitoes (Kline et al. 1990; Takken and Kline 1989), as well as biting midges (Bhasin et al. 2000; Blackwell et al. 1996; Harrup et al. 2012; Kline et al. 1994; Ritchie et al. 1994; Takken and Kline 1989). 1-Octen-3-ol is, however, not widely used in monitoring and control strategies against biting midges in Europe. Phenolic compounds, identified from cattle urine, have also been identified as attractants for tsetse flies (Bursell et al. 1988; Vale et al. 1988), and subsequently shown to attract biting midges (Bhasin et al. 2001; Kline et al. 1990). As for 1-octen-3-ol, few attempts have been made to incorporate these semiochemicals into control strategies (Bhasin et al. 2001; Cilek et al. 2003; Venter et al.2011).

Culicoides nubeculosus is a research model species with a similar host range as the known vector species of Bluetongue and Schmallenberg viruses in Europe (Lassen et al. 2011; Mellor and McCaig 1974; Nielsen and Christensen 1975; Pettersson et al. 2012). To date, *C. nubeculosus* has been used in susceptibility studies for these viruses (Jennings and Mellor 1988; Mellor 2000; Veronesi et al. 2013a, b). In the present study, we show the behavioral response of *C. nubeculosus* to individual cattle volatiles identified through combined gas chromatography and electroantennographic detection (GC-EAD) (Arn 1975) and combined GC and mass spectrometry (GC/MS) analyses.

Methods and Materials

Headspace Collection of Cattle Volatiles

Hair (15 g) from Holstein heifer cattle, taken from their back, neck, and belly area was placed in a 500 ml gas washing bottle (Lenz Laborglas, Wertheim, Germany). Charcoal-filtered air was drawn by a pump (Rena 301, Rena France S.A., Meythet, France), from the bottom to the top of the bottle, at 0.1 l min^{-1} over 24 h, passing through an adsorbent column containing 40 mg of Porapak Super Q (PQ; 80/100 mesh; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Five ml of urine, collected from the same cattle, were placed in a 250 ml gas wash bottle (Lenz Laborglas) and left in a 37 °C water bath to incubate overnight under aerobic conditions. An adsorbent column consisting of 50 mg PQ was used to trap volatiles from the cattle urine. Nitrogen was pushed into the wash bottle, through an internal dip tube placed ca. 0.5 cm from the urine surface, and through the adsorbent column connected to the exit tube. The flow rate through the column was 0.3 l min^{-1}, and the headspace was collected for 3 h. The adsorption columns were washed using *n*-hexane

(LabScan, Malmö, Sweden) and pentane (≥99.9, Merck KGaA, Darmstadt, Germany) prior to collection and eluted after collection using 500 µl of pentane.

Insects

*Culicoides nubeculosu*s were sent from The Pirbright Institute, UK as pupae, and received at our laboratory in Sweden as adults. Insects were maintained at 26 °C, 50 % RH, a 12:12 h L:D cycle, and provided with access to water from wet filter paper. One-to-4-d-old nulliparous, and presumed mated (Mair and Blackwell1996), female biting midges were used for GC-EAD and behavioral analyses. Females were visually separated from males by differences found in the antennal plumage (Blackwell et al. 1992); male antennae are covered by a large number of mechanosensory sensilla that gives the antenna a plumose appearance. At this age, females are activated by human breath, attracted to a human hand, and they would take a blood meal if given the opportunity.

Electrophysiology

For GC-EAD analysis, an Agilent 6890 gas chromatograph (GC; Agilent Technologies, Santa Monica, CA, USA) equipped with a fused silica capillary column (30 m x 0,25 mm) coated with 5 % phenyl-/95 % methylsiloxane (HP-5, film thickness = 0.25 µm; Agilent Technologies) was used. Hydrogen was the mobile phase ($Q = 45$ cm s^{-1}). Two µl of each sample were injected (splitless mode, 30 s, injector temperature 225 °C). The GC oven temperature was programmed from 30 °C (3 min hold), followed by a ramp of 8 °C min^{-1} to 225 °C, and then held isothermal for 10 min. At the GC effluent, 4 psi of nitrogen were added and split 1 : 1 in a Gerstel 3D/2 low dead volume four-way-cross (Gerstel, Mülheim, Germany) between the flame ionization detector and the EAD, via two 100 cm deactivated fused silica capillaries (0.25 mm ID; Agilent Technologies). The GC effluent capillary for the EAD passed through a Gerstel ODP-2 transfer line, that tracked the GC oven temperature, into a glass tube (10 cm x 8 mm), where it was mixed with charcoal-filtered, humidified air (1.5 l min^{-1}). The antenna was placed 0.5 cm from the outlet of this tube.

For EAD recordings, we modified the method described by Logan et al. (2009). In short, the head of a *C. nubeculosus* female was separated from the body, and the tip of each antenna was cut. Glass capillaries with a silver wire were filled with Beadle-Ephrussi ringer (Bjostad 1998). The recording glass electrode was placed over the tip of one antenna, and the reference electrode was inserted through the occipital opening with the tip positioned as close to the base of the antenna as possible. The EAD signal was pre-amplified (10 x) using a Syntech combi probe (Syntech, Kirchgarten, Germany). An IDAC2 (Syntech) interface converted the signals from the FID and EAD, and these signals were visualized using GC-EAD software v1.2.5 2014 (Syntech). Due to the instability of the antennal preparation, likely due to the large number of mechanosensory sensilla, we used the following criterion of a bioactive compound: an eluted compound that elicited an EAD response in at least three out of five female *C. nubeculosus*. EAD and FID traces were smoothed to reduce noise by averaging over 2 s in R (R Core Team 2014).

Chemical Analysis

The bioactive compounds in the headspace of cattle hair and urine detected by the antennae of *C. nubeculosus* were identified by injection on a combined 6890 N gas

chromatograph and 5975 mass spectrometer (GC/MS; Agilent Technology) fitted with a fused silica capillary column (30 m × 0,25 mm) coated with HP-5MS (film thickness = 0.25 μm), with the same temperature programme as that used for the GC-EAD analysis. Helium was used as the mobile phase ($Q = 35$ cm s^{-1}). The mass spectra were generated at 70 eV, and the bioactive compounds were identified by comparisons of their calculated Kováts indices and with reference spectra in a custom made data base and from commercially available mass spectral libraries (NIST05, Agilent Technology, and Wiley). The identified compounds were then confirmed by comparing mass spectra and retention times with those of commercially-available standards.

Behavioral Assay

A two choice behavioral assay, consisting of a Y-tube olfactometer (Fig. 1; arms 14 cm, stem 12.5 cm and inner Ø 2.2 cm; Humiglas, Södra Sandby, Sweden), was used to assess the behavioral activity of the headspace volatile extracts and the GC-EAD active compounds identified in the cattle hair and urine headspace. In addition, the behavioral response to octanal was assessed due to the structural resemblance of this compound to other tested compounds; this compound was also present in odor collections from cattle hair, but failed to meet the criterion of a bioactive compound. The Y-tube was placed inside a wooden box (310 x 400 x 450 mm), lined with white cloth, with an incandescent light source (230 V, 50 Hz, 0.40 A, 1170 lux) placed on top of the box, above the test arms. Synthetic air, containing metered 600 ppm CO_2 and oxygen (20 %), balanced by nitrogen (Strandmöllen AB, Ljungby, Sweden), was introduced, at a rate of 300 ml min^{-1}, into both arms using Teflon tubes via a glass tube (9.5, Ø 2.2 cm). The temperature and humidity in the room was set to 25 °C and 50 % RH, respectively.
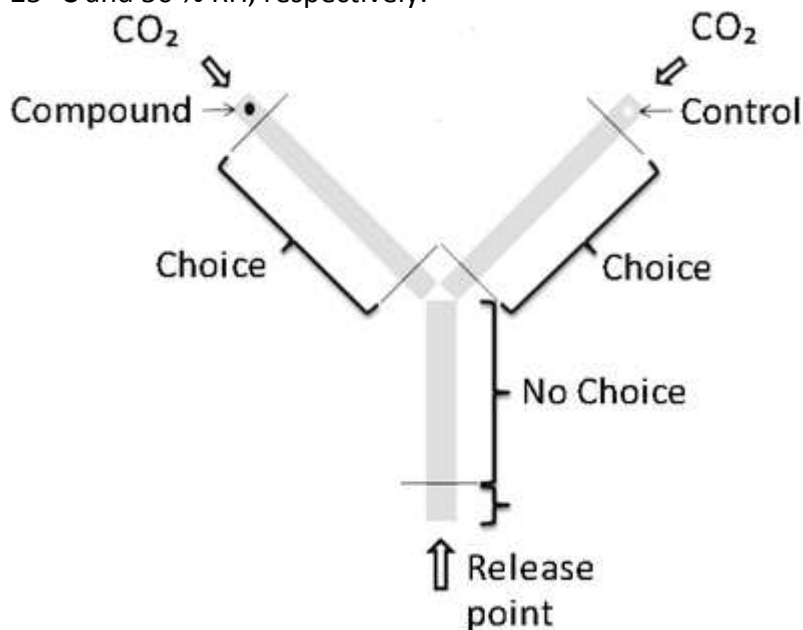

Fig. 1

Schematic drawing of the Y-olfactometer used to evaluate the behavioral response of *Culicoides nubeculosus* to the GC-EAD active compounds. Choice and no choice indicate the conditions under which the behavioral response was evaluated

Odor stimuli were produced by loading filter papers (1 x 1 cm) with undiluted headspace volatile extracts or solutions of test compounds (10 μl). Compounds (Table 1) were prepared

in redistilled *n*-hexane as serial solutions, with loadings on the filter paper ranging from 10^{-4}-10^{-12} g. Both headspace volatile extracts and the solutions were tested against hexane as a control. The solvent was allowed to evaporate for 30 s before the filter papers were attached to steel wires (1.5 cm long) that were positioned at the center of the glass tube at the control and test side of the Y-tube (Fig. 1). These glass tubes were separated from the arms via a mesh to prevent the insects from coming into contact with the release point. Ten to 15 female *C. nubeculosus* were released at the far end of the stem by fitting a 10 cm long glass tube to the stem containing the female insects, thereby introducing the females into the flow. Insects were allowed to make a choice for 7 min before the position of the individual insects in the olfactometer was recorded. Female *C. nubeculosus* that entered the treatment arm were considered to be attracted to the tested headspace volatile extract or compound solution, in combination with 600 ppm CO_2. In contrast, females that entered the control arm, containing the hexane control and 600 ppm CO_2, were considered inhibited by the tested extract or compound solution. Females that did not make an active choice, i.e., remained in the stem of the Y tube, were considered non-responders and were excluded from further analysis. Assays were performed between 8:00 and 10:00, i.e., at the host-seeking activity peak at dawn (Kettle 1962). Ten repetitions per compound and dose were performed. The olfactometer was washed with ethanol and heated at 300 °C for 8 h between treatments.

Table 1

Information on purity and manufacturer of the chemical compounds used in this study

Compound	Purity (%)	Supplier
Heptanal	>98	Sigma-Aldrich [a]
Octanal	>98	Sigma-Aldrich [a]
Nonanal	95	Sigma-Aldrich[a]
Decanal	>98	Sigma-Aldrich [a]
E-2-Nonenal	97	Sigma-Aldrich [a]
2-Phenylethanal	99.5	Sigma-Aldrich [a]
2-Ethylhexanol	>99	Sigma-Aldrich [a]
1-Octen-3-ol	98	Sigma-Aldrich [a]
6-Methyl-5-hepten-2-one	>98	Sigma-Aldrich [a]

Compound	Purity (%)	Supplier
3-Methylindole	98	Sigma-Aldrich [a]
Phenol	99	Sigma-Aldrich [a]
2-Methoxyphenol	95	Sigma-Aldrich [a]
3-Methylphenol	99	Sigma-Aldrich [a]
4-Methylphenol	99	Sigma-Aldrich [a]
3-Ethylphenol	99	Sigma-Aldrich [a]
4-Ethylphenol	99	Sigma-Aldrich [a]
3-Propylphenol	98	Alfa Aesar [b]
4-Propylphenol	99	Sigma-Aldrich [a]

[a]Sigma-Aldrich Chemie GmbH, Steinheim, Germany

[b]Alfa Aesar GmbH, Karlsruhe, Germany

Statistical analysis

Data analysis was performed to identify the concentrations of compounds tested that significantly attracted or inhibited the behavior of biting midges. For each replicate, the number of midges responding to the treatment was expressed as a proportion (0-1), calculated as the number of biting midges found in the treatment arm divided by the number of biting midges found in both treatment and control arms. Binomial logistic regression was then used to test whether the proportions choosing the test stimulus over 10 replicates differed significantly from that expected by chance (0.5). All data from bioassays were analyzed using R (R Core Team 2014).

Results

Electrophysiology and Chemical Analysis

Using GC-EAD analysis, we identified 9 constituents in the cattle hair (Fig. 2a) and 14 in the cattle urine headspace (Fig. 2b) that met our criterion of a bioactive compound. One compound, decanal, occurred in both extracts. Of these, we identified 6 and 12 of the EAD active compounds in the cattle hair and urine headspace, respectively, using GC/MS analysis

(Table 2). We acknowledge that the use of pentane as a solvent is selective with respect to the polarity of the eluted compounds.
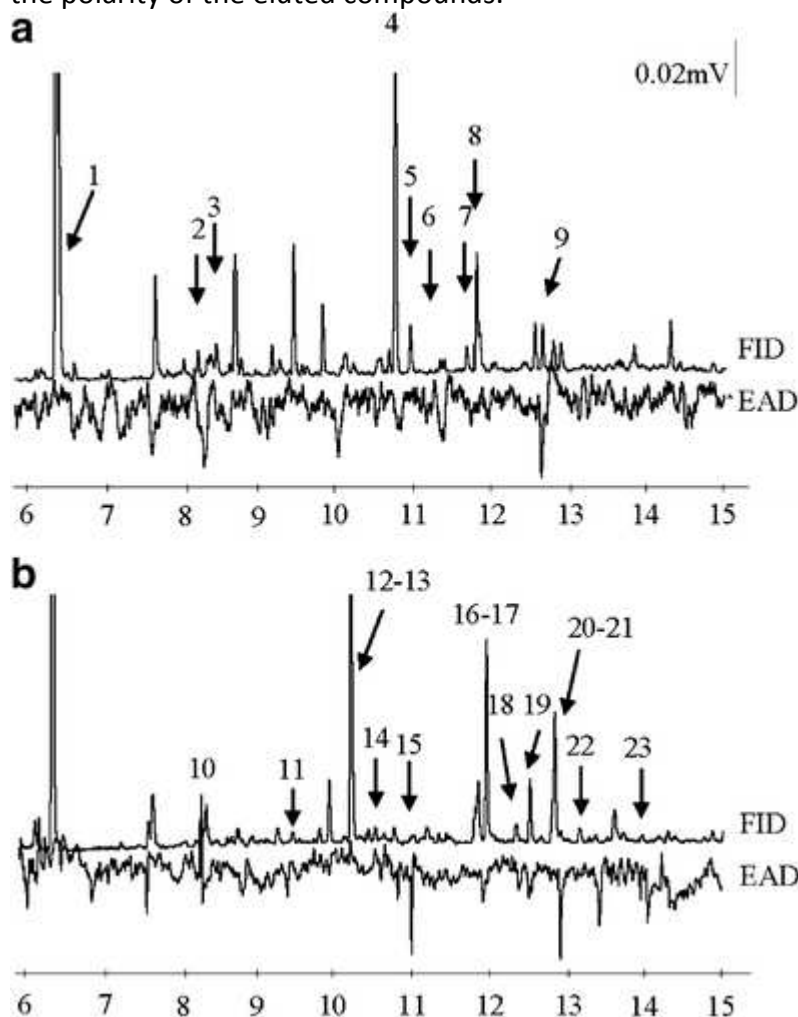

Fig. 2

Antennal response of *Culicoides nubeculosus* to cattle hair and urine volatiles. Combined gas chromatography and electroantennographic detection (GC-EAD) signal from the female antenna to compounds from cattle hair (**a**) and urine (**b**) eluting from an HP-5 capillary column. EAD signal in (b) is an average of two runs. Top traces correspond to the signal of the flame ionisation detector (FID) of the GC, and the bottom traces to the electroantennographic (EAD) response. Peak number: 1. Heptanal, 2. 1-Octen-3-ol, 3. 6-Methyl-5-hepten-2-one, 4. Nonanal, 8. *E*-2-Nonenal, 9. Decanal, 10. Phenol, 11. 2-Ethylhexanol, 12-13. 3- and 4-Methylphenol, 14. 2-Methoxyphenol, 15. 2-Phenylethanal, 16-17. 3- and 4-Ethylphenol, 18. Decanal, 20-21. 3- and 4-Propylphenol, 23. 3-Methylindole, 5-7, 19 and 22. Unknown. Differences in retention times of decanal are due to slightly different GC-conditions

Table 2
Combined gas chromatography and mass spectrometry (GC/MS) identification of compounds in headspace volatile extracts of cattle hair and urine eliciting electroantennographic activity in *Culicoides nubeculosus*. KI: Kováts index. The peak numbers correspond to the peak numbers in Fig 2

	Peak	KI	Compound
	1	898	Heptanal
	2	977	1-Octen-3-ol
	3	986	6-Methyl-5-hepten-2-one
	4	1107	Nonanal
	5	1119	Unknown
	6	1139	Unknown
	7	1150	Unknown
	8	1168	*E*-2-Nonenal
Cattle hair	9	1212	Decanal
	10	978	Phenol
	11	1032	2-Ethylhexanol
	12	1076	4-Methylphenol
	13	1076	3-Methylphenol
	14	1096	2-Methoxyphenol
	15	1119	2-Phenylethanal
	16	1143	4-Ethylphenol
	17	1143	3-Ethylphenol
Cattle urine	18	1212	Decanal

	Peak	KI	Compound
	19	1259	Unknown
	20	1269	4-Propylphenol
	21	1269	3-Propylphenol
	22	1304	Unknown
	23	1411	3-Methylindole

Behavioral Assay

The headspace odor of cattle hair ($Z = 2.34$, $df = 1$, $P < 0.05$) and urine ($Z = 2.61$, $df = 1$, $P < 0.01$), when combined with CO_2, significantly attracted host-seeking *Culicoides nubeculosus* over that of CO_2 alone (Fig. 3), in the two-choice assay. The GC-EAD active compounds and octanal elicited attraction, inhibition, or non-preference depending on the compound and concentration tested (Fig. 4). Decanal (when tested at 10^{-10} and 10^{-4} g), 2-phenylethanal (10^{-8} g), 2-ethylhexanol (10^{-8} and 10^{-6} g), 1-octen-3-ol (10^{-8} g), 3-methylindole (10^{-10} and 10^{-8} g), phenol (10^{-8} g), and 3-ethylphenol (10^{-10} g), when combined with 600 ppm CO_2, elicited a significant attraction of *C. nubeculosus* compared to CO_2 alone. In contrast, heptanal (10^{-4}g), octanal (10^{-10} g), nonanal (10^{-10} g), *E*-2-nonenal (10^{-6} and 10^{-4} g), 3-propylphenol (10^{-8}, 10^{-6}, and 10^{-4} g), and 4-propylphenol (10^{-10}, 10^{-6} and 10^{-4} g), when combined with 600 ppm CO_2, elicited behavioral inhibition. Both attraction (a) and behavioral inhibition (i) was observed when testing 6-methyl-5-hepten-2-one (a: 10^{-10}g, i: 10^{-8} g), 3-methylphenol (a: 10^{-8} g, i: 10^{-6} g), 4-methylphenol (a: 10^{-8} g, i: 10^{-4} g), and 4-ethylphenol (a: 10^{-8} g, i: 10^{-4} g), in combination with 600 ppm CO_2. 2-Methoxyphenol did not elicit any significant behavioral response when compared to the CO_2 control.
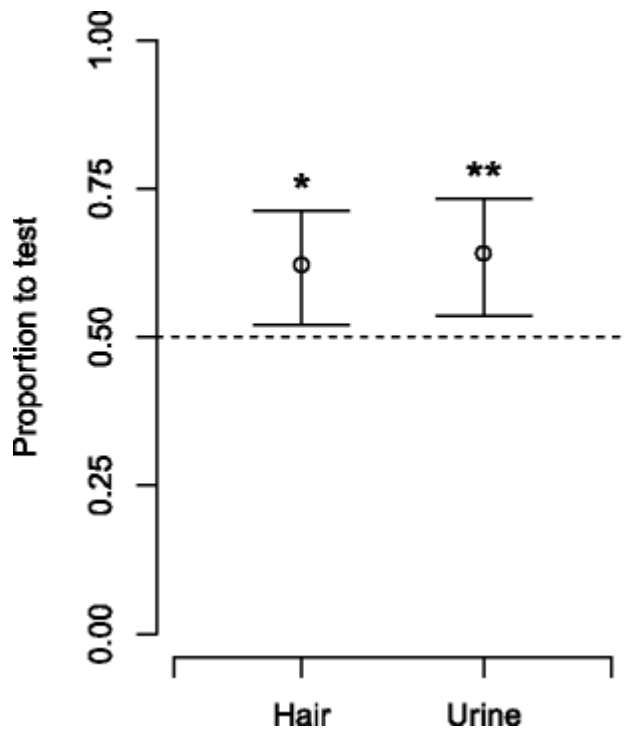
Fig. 3

Estimated proportions (+/- 95 % confidence intervals) of female *Culicoides nubeculosus* choosing the test side in the Y olfactometer with headspace odor of cattle- hair or urine. The dotted line indicates the proportion expected by chance (0.5). Asterisks indicate proportions differing significantly from 0.5 (* < 0.05, ** < 0.01)
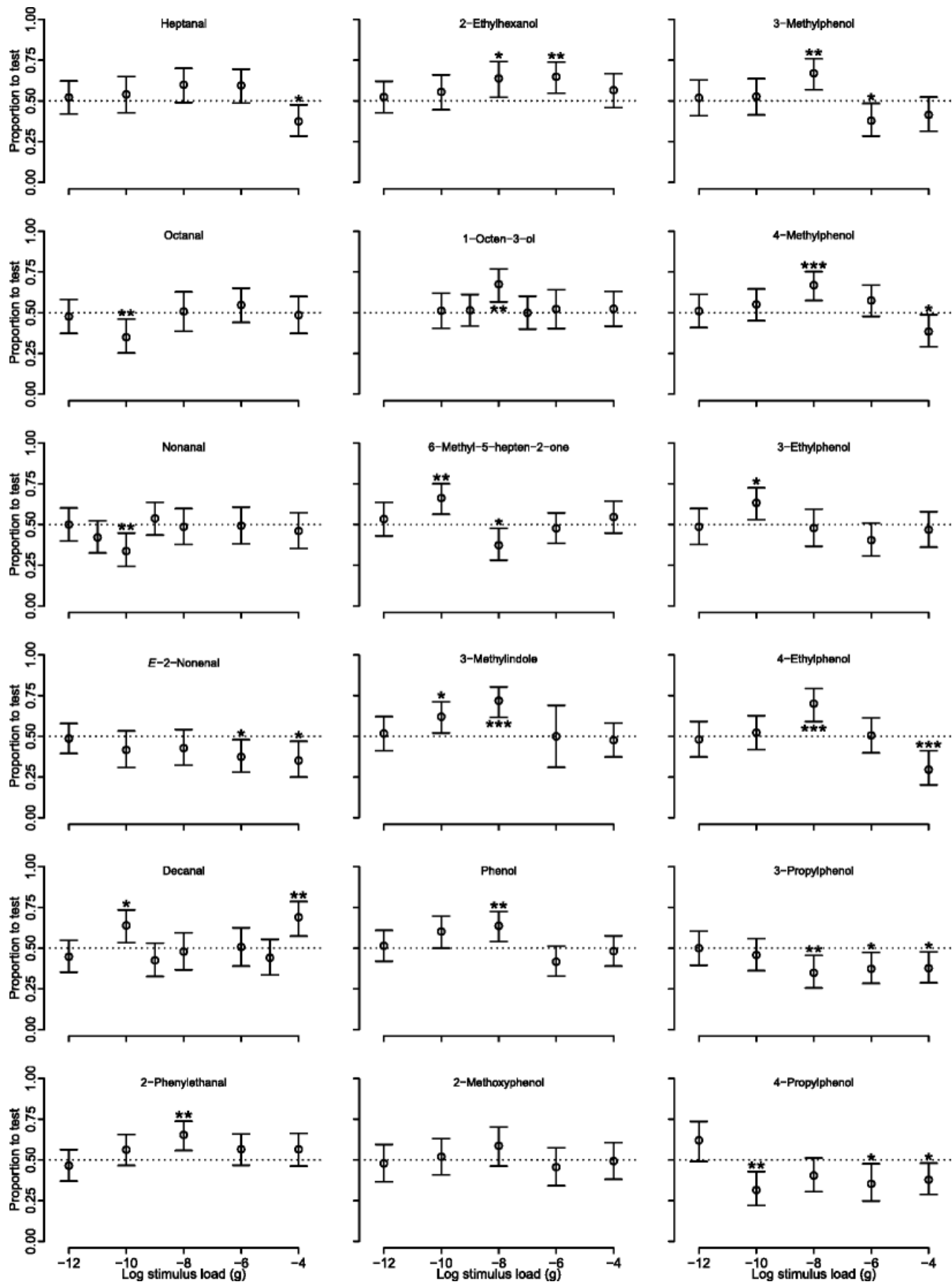
Fig. 4

Estimated proportions (+/- 95 % confidence intervals) of female *Culicoides nubeculosus* choosing the test side in the Y olfactometer with different concentrations of the electroantennographically active compounds (*N* = 10 for each concentration). The

dotted line indicates the proportion expected by chance (0.5). Asterisks indicate proportions differing significantly from 0.5 (* < 0.05, ** < 0.01, *** < 0.001)

Discussion

Due to the introduction and increased incidence of diseases vectored by *Culicoides* biting midges, there is a dire need to develop and standardize monitoring and control methods to manage these insects. Following the successful use of host-odor baited traps to control and survey other haematophagous insects (Logan and Birkett 2007; Pickett et al. 2010) we here aimed at identifying natural host volatiles that modulate the host-seeking behavior of *Culicoides* biting midges. We show, for the first time, that volatiles of cattle hair and urine, identified through GC-EAD and GC/MS analyses, elicit both attraction and behavioral inhibition in *C. nubeculosus*. Moreover, we show that the behavioral tuning in general is narrow, i.e., female *C. nubeculosus* often respond to a narrow range of concentrations of the individually tested compounds. This may have implications for their use in modulating the behavior of *Culicoides* biting midges in further field studies.
1-Octen-3-ol, heptanal, octanal, nonanal, decanal, *E*-2-nonenal, and 6-methyl-5-hepten-2-one, identified in the headspace of cattle hair (Birkett et al. 2004; Gikonyo et al. 2002; Tchouassi et al. 2013), are detected by the peripheral olfactory system of *Culicoides* biting midges (Bhasin et al. 2000; Blackwell et al. 1996; Logan et al. 2009), as well as of other haematophagous insects, including mosquitoes (Ghaninia et al. 2008; Logan et al. 2008; Syed and Leal 2009), tsetse flies (den Otter et al. 1988; Gikonyo et al. 2002), bed bugs (Harraca et al. 2012), and triatomine bugs (Guerenstein and Guerin 2001). Similarly, phenol, 3-methylphenol, 4-methylphenol, 4-ethylphenol, and 2-ethylhexanol, identified in cattle urine headspace collections (Bursell et al. 1988), elicit antennal responses in *C. impunctatus* (Bhasin et al. 2000; Logan et al. 2009). Several of the phenolic compounds also elicit antennal responses in mosquitoes (Hill et al. 2009; Qiu et al. 2006; Siju et al.2010) and tsetse flies (den Otter 1991). Accumulating evidence thus suggests that the olfactory systems of haematophagous insects have evolved convergently to respond to a number of generic host volatiles, and even blends of these (Guidobaldi and Guerenstein 2013), and that these may be exploited to increase trap captures for control and surveillance purposes across taxa.
The generic mammalian volatile, 1-octen-3-ol has been assessed extensively as a behavioral attractant of *Culicoides* biting midges, as well as of other haematophagous insects (Logan and Birkett 2007). In this study, we showed that *C. nubeculosus* are attracted to 1-octen-3-ol when presented at 10^{-8} g, a concentration considerably lower than that found in previous studies on *C. nubeculosus* (10^{-4}-10^{-3} g) (Bhasin et al. 2000) and on *C. impunctatus* (10^{-5}-10^{-1} g) (Bhasin et al. 2000; Blackwell et al. 1996). Differences in experimental design, particularly the inclusion of CO_2 in the present study, likely account for the difference in results between these studies. The other alcohol identified in this study, 2-ethyl-1-hexanol, also elicited attraction of female *C. nubeculosus*. It is noteworthy that 2-ethyl-1-hexanol is a precursor of the common plasticiser dioctyl phthalate (Thorat et al. 1992) and may, therefore, be an artefact or pollutant. However, 2-ethyl-1-hexanol occurs naturally in plants (Bruce and Pickett 2011).
Host-derived aldehydes play a role in the sensory ecology of various haematophagous arthropods (Gikonyo et al. 2003; Harraca et al. 2012; Logan et al. 2009; Syed and Leal 2009; Tchouassi et al. 2013). For mosquitoes, aldehydes appear to balance attraction and

behavioral inhibition depending on their relative ratio in complex host odor blends, as shown in both laboratory assays and field experiments (Logan et al. 2008; Tchouassi et al. 2013). With the exception of decanal and 2-phenylethanal, the aldehydes identified in this study elicited behavioral inhibition, either at physiologically relevant concentrations (10^{-10} g for octanal and nonanal) or at the highest concentrations tested (10^{-4} for heptanal, and 10^{-6} and 10^{-4} for E-2-nonenal). Although further studies are required to confirm the role of these host-derived aldehydes in regulating intraspecific host selection, the observed behavioral responses are in line with those observed previously (Logan et al. 2008, 2009; Tchouassi et al. 2013).

The ketone, 6-methyl-5-hepten-2-one, has also been shown to regulate differential attraction of $C. impunctatus$ (Logan et al. 2009) and of the mosquito $Aedes\ egypti$ (Logan et al. 2008) to humans, as well as of the horn fly, $Heamatobia\ irritans,$ to cattle (Birkett et al. 2004). Similar to these studies, we observed attraction, behavioral inhibition or non-preference depending on the concentration tested. As for the aldehydes, further studies are required to comprehend fully the behavior evoked in biting midges by 6-methyl-5-hepten-2-one, particularly in the context of a more complex host odor blend.

Phenolic compounds present in aged cattle urine have played a significant role in control strategies of tsetse flies (Vale and Torr 2004). Presented as blends at natural release rates, these compounds increase the trap catch of tsetse flies compared to individual compounds (Bursell et al. 1988; Vale et al. 1988). Our study showed that phenol, 3-ethylphenol, 4-ethylphenol, 3-methylphenol, and 4-methylphenol, when released within their natural concentration range (Torr et al. 1995), and in combination with CO_2, attract $C. nubeculosus$. In contrast, when presented at high concentrations, most of the phenolic compounds inhibit the behavioral response of $C. nubeculosus,$ which is in line with that found by Bhasin et al. (2000) for $C. impunctatus$. We note that the behavioral tuning to the methyl- and ethylphenols on one hand and the propylphenols on the other are largely aligned. Whether these compounds elicit distinct responses in different functional types of olfactory sensory neurons, which could explain how different behavioral circuits can be activated, remains for future studies. In addition, future studies will have to evaluate the ecological relevance of the behavioral response of $C. nubeculosus$ to urine-derived compounds.

Acknowledgments

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

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