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Medical and Veterinary Entomology

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https://doi.org/10.1111/mve.12622

Received: 22 July 2022 | Accepted: 17 October 2022

DOI: 10.1111/mve.12622

ORIGINAL ARTICLE

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Fermenting molasses and a synthetic odour blend to attract blood-fed *Anopheles coluzzii*

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Funding information
Nederlandse Organisatie voor Wetenschappelijk Onderzoek, Grant/Award Number: 863.13.012; Swiss Federal Food Safety and Veterinary Office

Abstract

Collecting blood-fed mosquitoes to monitor pathogen presence or to gather information on the host blood meal is often challenging. Fermenting molasses can be used to produce carbon dioxide to attract host-seeking mosquitoes, however, earlier work indicated that it may also attract blood-fed mosquitoes in the field. In the current study, these field results were validated in an experimental setting using a large cage setup with *Anopheles coluzzii* (Diptera, Culicidae). Blood-fed mosquitoes were indeed attracted to fermenting molasses with the highest attraction at 72 hours post feeding, which was used for subsequent experiments. Next, it was tested if fermentation of molasses is required for attraction, and whether it acts as an oviposition attractant, increases egg laying, or increases mosquito survival. The compounds that could be responsible for attraction were identified by combined electrophysiology and chemical analyses and formulated into a synthetic blend. Fermenting molasses attracted blood-fed mosquitoes in the large cage study, while fermenting sugar and non-fermenting molasses did not. The fecundity of blood-fed mosquitoes increased after feeding on fermenting molasses, however, compounds emanating from molasses did not trigger oviposition. The synthetic blend attracted blood-fed mosquitoes and may be used to determine mosquito host selection and for xenomonitoring, as 'flying syringes' to detect non-vector borne pathogens.

KEYWORDS
host feeding, malaria, oviposition, sugars, vector-borne diseases, xenodiagnostics, yeast

INTRODUCTION

When searching for a host, mosquitoes may express an opportunistic feeding behaviour or a more specific preference for certain (groups of) species, such as reptiles, birds or mammals (Wolff & Riffell, 2018). This host preference is an important determinant of the vectorial capacity of a mosquito species; for example, the highly anthropophilic mosquito species *Anopheles gambiae* sensu stricto and *Aedes aegypti* comprise two of the most important vectors of human pathogens (Takken & Knols, 2010; Takken & Verhulst, 2013). However, whether a host will be bitten is not only determined by the host preference of the mosquito, but also by other factors, such as host availability and abundance, which may lead to selection of a host that is different from the preferred host (Takken & Verhulst, 2013; Wooding et al., 2020; Yan et al., 2021). Since host selection plays an important role in pathogen transmission, it is critical to collect blood-fed mosquitoes and determine their bloodmeal host. In addition, the collection of blood-fed mosquitoes can be used for so-called xenodiagnostics or xenomonitoring, whereby the blood meal of the mosquito can be used to detect (non-vector borne) pathogens or their antibodies (Cameron & Ramesh, 2021; Fauver et al., 2017; Štefanić et al., 2022). The development of xenomonitoring systems with blood-fed

mosquitoes, may contribute to early warning systems for emerging infectious diseases (Cameron & Ramesh, 2021). Unfortunately, current methods for collecting blood-fed mosquitoes are inefficient and often biased. For example, catching blood-fed mosquitoes inside houses, followed by determining the origin of their bloodmeals, is biased because catches are done near the host inside the house (Fikrig & Harrington, 2021; Takken & Verhulst, 2013). Alternatives for the collection of blood-fed mosquitoes can be the use of resting boxes (Qiu et al., 2007), gravid traps (Opoku et al., 2018), pit shelters (Kweka & Mahande, 2009), or barrier screens (Keven et al., 2021), deployed either indoors or outdoors in which (blood-fed) mosquitoes become trapped (Burkot et al., 2013). However, catch rates with these methods are often low, which can be a result of trap type or other factors, such as mosquito density, behaviour, trapping times, placement of the trap, weather conditions, and so forth.

When catching host-seeking mosquitoes, carbon dioxide (CO_2) is an important cue and is often used as an attractant (Cardé, 2015; Coutinho-Abreu et al., 2021). Interestingly, when using fermenting molasses as a cheaper alternative to sugar fermentation to produce CO_2, blood-fed mosquitoes are also caught. In a study by Mweresa et al. (2014), the number of blood-fed *A. gambiae* sensu *lato* caught outdoors with MM-X traps was about four times higher than the number of blood-fed mosquitoes caught with fermenting sugar, and not significantly different from the number of unfed mosquitoes caught.

As it remained unknown which factors contributed to these captures, we aimed to validate the field results in a laboratory large cage setup with *Anopheles coluzzii* (Diptera, Culicidae) mosquitoes. Next, to understand the mechanism underlying why blood-fed mosquitoes are attracted to fermenting molasses, we tested whether fermenting molasses (i) acts as an oviposition attractant, (ii) increases oviposition and/or (iii) increases mosquito survival. Finally, a synthetic fermenting molasses odour blend was identified by using combined gas chromatography (GC) and electroantennographic detection (GC–EAD), together with combined GC and mass spectrometer (GC–MS) analyses. This synthetic lure was assessed in olfactometer experiments to determine the behavioural response of blood-fed mosquitoes.

MATERIALS AND METHODS

Mosquitoes

The laboratory colony of *A. coluzzii* originated from Suakoko, Liberia and was maintained at 27 ± 1°C and 80 ± 5% relative humidity with a photo-scotophase of 12:12 h. Adults were kept in cages (30 × 30 × 30 cm^3, Bugdorm, MegaView Science, Taiwan) and provided 6% glucose solution. Mosquitoes were fed daily with human blood (Sanquin Blood Bank, The Netherlands) through a Hemotek PS5 membrane feeder (Hemotek Ltd., UK) set at 38°C, with the addition of 5% CO_2 and a worn sock to mimic a human host. In the cages, females could lay eggs on wet filter paper and afterwards the eggs were transferred to trays with water, supplemented with Liquifry No. 1 (Interpet Ltd., UK), before hatching. Larvae were fed daily with Tetramin fish food (Tetrawerke, Germany). Pupae were removed from the trays and allowed to emerge inside the Bugdorms used for maintaining adults. Adult female mosquitoes of 5–8 days post-emergence were collected and used for experiments, unless indicated otherwise. Adult mosquitoes for the GC–EAD analysis were reared similarly with minor modifications as described by Omondi et al. (2015).

Preparation of fermenting molasses and fermenting sugar

Mixtures were made according to Smallegange et al. (2010): 250 g sugar (Van Gilse Kristalsuiker, Suiker Unie, The Netherlands) or 250 g molasses (Mulder Agro, The Netherlands) was mixed with 17.5 g yeast (Bio Speciaal, The Netherlands) and 2 L tap water. The components were mixed in 5 L plastic containers by shaking them for 30 s under ambient conditions (Mweresa et al., 2014). Mixing took place 1–1.5 h prior to releasing the mosquitoes into the bioassays, to ensure sufficient CO_2 production during the experiments.

Attractiveness of fermenting molasses to blood-fed mosquitoes

A single-choice experiment was performed to test at which moment after blood-feeding mosquitoes were most attracted to fermenting molasses, and to confirm previous results obtained in the outdoor experiments by Mweresa et al. (2014). One group of 280 mosquitoes was offered a blood meal whereas another group of 200 mosquitoes was not blood-fed. Female mosquitoes used were at least 5 days post-emergence, to ensure that all had mated, and a maximum of 8 days post-emergence. Mosquitoes that did not take a bloodmeal were removed from the cage. All mosquitoes were provided with a 6% glucose solution until their release. Experiments were performed in a 3.0 × 3.0 × 3.0 m^3 textile screen cage in a climate-controlled room (25 ± 1°C and 75 ± 5% relative humidity) (Smallegange et al., 2010). Inside the cage, a BG-Suna trap (Biogents AG, Germany) (Hiscox et al., 2014), which contained a sock previously worn for 12 h (40 Den, 100% polyamide, HEMA, The Netherlands) providing human odour, was placed in the middle of the cage at a height of 30 cm. The 5 L plastic container with either fermenting molasses or sugar was placed next to the BG-Suna trap, with a tube connected to the CO_2 connection of the trap. In the first trial, fermenting sugar was used as an attractant and in a second trial, fermenting molasses was used. Mosquitoes were tested at 24, 48 and 72 h after blood-feeding. At each time point, a group of 50 blood-fed and 50 non-blood-fed mosquitoes were released in the textile cage. Since the blood would largely be digested by the mosquitoes during the trial, the groups that were released 72 h after the start were colour-marked in order to distinguish between blood-fed and non-blood-fed mosquitoes. These were marked using fluorescent powder as described previously, which has no effect on mosquito behaviour (Verhulst et al., 2013).

Before, during and after releasing mosquitoes, the concentration of CO_2 was measured to exclude variation in CO_2 production as a factor explaining the observed effects. The concentration was measured by placing a CO_2 meter (Vaisala CARBOCAP® Hand-Held CO_2 Meter GM70) 5 cm inside the CO_2 tube of the BG-Suna trap.

Around 2 h after releasing the mosquitoes in the textile screen cage, the BG-Suna trap and release cages were closed. Mosquitoes were killed by freezing in order to count the trapped mosquitoes and mosquitoes that did not leave the release cages. The mosquitoes in the textile screen cage that were not caught in the trap were recaptured by mouth aspiration. The number of mosquitoes which left the release cages were subtracted from the total number of mosquitoes. After freezing, the mosquitoes were counted, and their abdominal status (blood-fed or non-blood-fed) determined.

In a second experiment it was determined whether fermentation is required to attract blood-fed mosquitoes. Procedures were similar to those described with both fermenting molasses and fermenting sugar being retested. In addition, mixtures of water and molasses or water and sugar were assessed with the yeast kept separately nearby, so that no fermentation occurred. Groups of 50 mosquitoes were released 72 h after blood-feeding. Each treatment was tested four times on four different days according to a 4×4 Latin square design.

All experiments were performed while wearing gloves to prevent human odour contamination. In between experiments, traps were cleaned with 70% ethanol and aired to remove any residual odours.

Molasses as an oviposition attractant

Dual-choice oviposition experiments were performed in Bugdorm cages located ina climate-controlled room ($25 \pm 1°C$ and $75 \pm 5\%$ relative humidity) to establish whether fermenting molasses may act as an oviposition attractant to female *A. coluzzii*. Each cage contained one blood-fed mosquito that was fed 24 h in advance. Each cage contained a vial of 6% glucose solution and four oviposition cups with two different treatments (Figure S1). Treatments were randomized among and within the cages. The oviposition cups consisted of a plastic cup (height: 3 cm, diameter: 5 cm) and an inner cup (height: 2.25 cm, diameter: 2.25 cm) used to prevent the oviposition paper getting into direct contact with the treatment in the oviposition cup. The wet filter paper, on which the mosquitoes could lay their eggs, was placed inside the water-filled inner cup (Figure S1).

Cups were filled with 0.175 g yeast and 20 ml water, together with either 2.5 g sugar (fermenting sugar treatment) or 2.5 g molasses (fermenting molasses treatment). The control oviposition cups were filled with tap water only. Fresh fermenting molasses, sugar and water were provided daily to each cage. Each day, until 94 h after blood-feeding, eggs laid on the filter papers were counted. Each treatment combination was tested 14 times.

Molasses as a factor affecting fitness

Experiments were conducted to assess whether molasses acts as a nutritional source affecting the number of eggs laid or enhance adult survival. To determine the effect of different treatments on egg laying, blood-fed mosquitoes were offered a fermenting molasses solution, a fermenting sugar solution or water alone as a control. Before blood-feeding, mosquitoes (male and female) were kept together in a cage and provided a vial with a 12.5% glucose solution for 4 days. On Day 5, groups of 60 female mosquitoes were transferred to three Bugdorm cages with the different solutions and provided with blood for 2 h, as described above. The mosquitoes that did not feed on the blood were enumerated and removed from each cage. The blood-fed mosquitoes were kept in the cage for two additional days and provided with fresh fermenting molasses, sugar or water daily. Thereafter, 20 blood-fed females were selected from each cage and transferred to individual 50 ml Falcon tubes for 72 h for egg laying. The oviposition tubes contained a few millilitres of water to wet the filter paper on which the mosquitoes could lay eggs. The respective treatments (fermenting molasses, fermenting sugar and water) were also provided to the individual mosquitoes in the oviposition tube through a piece of cotton with a strip of towel on top that were both moistened with the treatment. Freshly treated cotton and towels were provided daily. Five replicates of the three different mosquito groups were carried out for all treatments. Egg laying and survival of individual mosquitoes in their oviposition tubes was recorded for 6 days.

Headspace volatile collection

Headspace samples were prepared in a 5 L polyamide bag (Toppits, Cofresco, Germany), kept in a 5 L bucket, by mixing 125 g molasses (Granngården, Sweden) or sugar (DanSukker, Sweden) and 100 ml distilled water, and then 8.75 g dry yeast (Kron Jäst, Sweden) was added to the mix. For the control, 125 g molasses or sugar and 100 ml distilled water was mixed, and the dry yeast was suspended in a fine-mesh sachet (100 μm mesh size) above the mix. In addition, an empty bag was used as a negative control. Mixing took place 2 h before headspace sampling, at room temperature, to ensure fermentation. A charcoal-filtered continuous airstream (0.5 L min^{-1}) was pushed into the bag by a diaphragm vacuum pump (KNF Neuberger, Germany), over the sample and into an aeration column, in a closed loop, for 3 h. The aeration column was made of Teflon tubing (6 cm $\times$ 3 mm internal diameter), filled with 30 mg of Porapak Q (50/80 mesh Alltech Associates, Inc., USA) between polypropylene wool plugs and Teflon stoppers. The columns were rinsed with 1 ml dichloromethane (Merck, Germany), 1 ml redistilled *n*-hexane (Merck) and 2 ml pentane (puriss pro ana; Sigma-Aldrich Chemie GmbH, Germany) prior to use. Adsorbed volatiles were eluted with 400 μl pentane. For both treatment and controls, five replicates were collected. The headspace samples were stored at −20°C in 4 ml glass vials until further analyses.

TABLE 1 The compounds identified in the gas chromatography–electroantennographic detection analysis and used for the synthetic blend

Compound name	Chemical Abstract Service (CAS) num	Purity (%)	Retention time (N)	Kovats' index	Reference (Nist)	Peak area	Synthetic blend (ng/µl)
Isoamylacetate	123-92-2	≥97	7.243	867.89	867	15,129,098	4.5
Styrene	100-42-5	≥98	7.619	888.81	889	117,757,074	35.0
Alpha-pinene	80-56-8	≥98	8.514	935.05	935	3,665,071	1.0
2-Ethyl-6-methyl pyrazine[a]	13925-03-6		9.767	998.33	998	23,611,779	–
2,3,5-Trimethylpyrazine	14667-55-1	≥99	9.859	998.64	990	29,177,107	8.8
Ethyl caprylate	106-32-1	≥98	12.015	1192.49	1192	41,365,682	12.3
Caprolactam	105-60-2	99	13.552	1267.95	1252.9	38,407,420	11.5
Ethyl decanoate	110-38-3	≥98	14.916	1396.52	1396	9,304,439	2.8

Note: Concentrations used in synthetic blend are based on the gas chromatography peak areas, as compared to the internal standard.
All compounds were ordered from Sigma-Aldrich, Germany.
[a]Not included in the synthetic blend because it was not commercially available.

Electrophysiological and chemical analyses

Bioactive volatile organic compounds within the headspace of fermenting molasses and sugar were identified using GC–EAD analysis, as previously described (Wondwosen et al., 2016). In short, the head of a blood-fed *A. coluzzii* was excised, and a pulled glass microcapillary, serving as the reference electrode, filled with Beadle–Ephrussi ringer solution, was inserted into the foramen. The distal segment of the antenna was then cut and inserted into a recording electrode filled with the same ringer solution. The recording electrode was connected to a pre-amplifier (10×), which in turn was connected to a high impedance DC amplifier interface box (IDAC-2; Syntech, Germany). After mounting the antenna, 2 µl of a pooled extract, comprising all headspace collections, of fermenting molasses or sugar was injected onto the GC (Agilent Technologies 6890 GC, USA), equipped with an HP-5 column (30 × 0.25 mm^2 i.d., fused silica, 0.25 µm film thickness, Agilent Technologies) in splitless mode (30 s, injector temperature 225°C). Hydrogen was used as the mobile phase at an average linear flow rate of 45 cm/s. The GC oven temperature was set from 40°C (3 min hold) at 10°C/min to 250°C (5 min hold). The GC effluent was split 1:1 in a Gerstel 3D/2 low dead volume four-way cross (Gerstel, Germany) between the flame ionization detector and the EAD. The GC effluent for the EAD passed through a Gerstel ODP-3 transfer line, which tracked the GC oven temperature, into a glass tube (10 cm × 8 mm), where it was mixed with charcoal-filtered, humidified air (1.5 L/min). The distal end of this glass tube was placed 0.5 cm from the antennal preparation. Three consistent replications were collected and analysed using GC–EAD 2011 software (V.1.2.3, Syntech, Kirchzarten, Germany).

The bioactive volatile organic compounds in the pooled headspace extract of fermenting molasses and sugar determined through GC–EAD analysis were identified using a GC–MS (6890GC and 5975MS or 7890GC and 5975MS; Agilent Technologies), operated in the electron impact ionization mode at 70 eV. The GC was equipped with either of two fused silica capillary columns (30 m × 0.25 mm i.d. × 0.25 µm film thickness) coated with DB-Wax or HP-5 (Agilent Technologies). Helium was used as carrier gas at a linear flow rate of 35 cm/s. Two microlitres of either the pooled extract of fermenting molasses or sugar and the two controls were injected. The GC oven temperature program was the same as for the GC–EAD analysis. Compounds were identified according to Kovats' retention index and mass spectra, in comparison to custom-made and NIST14 libraries (Agilent). The identified bioactive compounds of fermenting molasses were confirmed by co-injection of authentic standards (Table 1). Co-injection of an internal standard, 100 ng heptyl acetate (99.8%; Merck, Solna, Sweden) was used for quantification.

Attractiveness of a synthetic blend in an olfactometer

The bioactive compounds identified from the fermenting molasses were combined in a synthetic blend and tested in a triple layered, dual-choice olfactometer for their attractiveness to blood-fed *A. coluzzii*. The synthetic blend consisted of seven compounds diluted in pentane in different ratios, based on the area under the peak, as determined by the GC–MS analysis (Table 1). The 2-Ethyl-6-methyl pyrazine was not included as it was not commercially available. The synthetic odour blend was tested in a dose-dependent manner, using five doses in half decadic steps (but same ratios). The synthetic blend was released from wick dispensers, using 1.5 ml glass vials (GeneTec AB, Sweden), with wicks constructed of 5 cm × 1.5 mm Teflon tubing lined with a cotton thread (Safran Drops, The Netherlands) inserted through the lids (Birgersson et al., 2012). Each vial contained 1 ml of the synthetic blend and was stored at −20°C between experiments. Fermenting molasses was included as a positive control and prepared as described for the large cage experiments.

Experiments in triple layered, dual-choice olfactometers (each layer measuring 1.60 × 0.66 × 0.43 m^3) were performed as described by Verhulst et al. (2018). Blood-fed mosquitoes were released in the olfactometer 72 h after the bloodmeal in groups of 35. The trapping

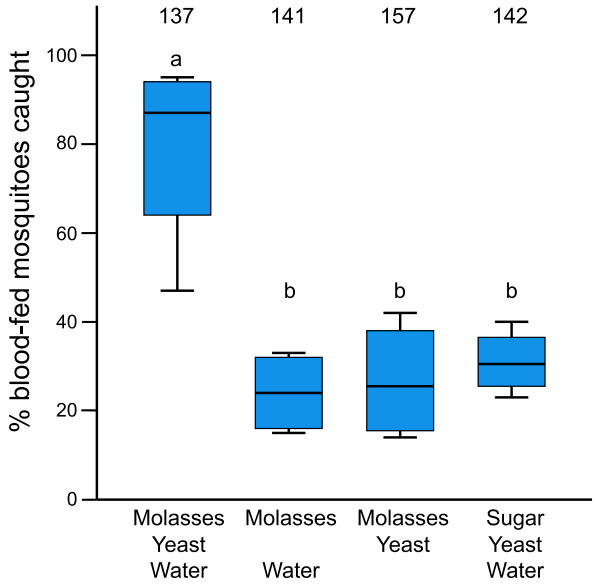

FIGURE 1 Effect of fermenting molasses and sugar on the percentage of blood-fed mosquitoes caught in large cage experiments. Mosquitoes were fed 72 h in advance. Numbers on top indicate the number of mosquitoes that left the release cage. Different letters above the boxplots indicate statistically significant differences (generalized linear model, $df = 9$, $p < 0.05$)

devices on the upwind side of the olfactometer contained a vial with the synthetic blend or a control with the solvent only. The fermenting molasses was tested by inserting a 1.5 m silicon tube from the fermenting 5 L container into the trapping device of the olfactometer. In this case, the control was an empty trapping device. Each treatment was repeated six times and the samples were alternated between trapping devices and olfactometers to minimize positional effects.

Statistics

Differences in the CO_2 production between fermenting molasses and fermenting sugar at different time points were tested with a linear mixed model (LMM) with time as repeated measure, after verifying the normal distribution of the data. To test at which moment after blood-feeding mosquitoes were most attracted to fermenting molasses, *Z*-tests were used to compare the number of mosquitoes that were captured when exposed to the different CO_2 sources.

A generalized linear model (GLM, binomial distribution, logit link function) was used to analyse the differences in trap catches in large cage experiments when assessing whether fermentation is required for the attraction of blood-fed mosquitoes. Parameters fitted in this model were the effect of day of release, temperature, relative humidity and time of release. Differences in the number of eggs laid in the two-choice oviposition experiments were analysed with a Wilcoxon signed rank test. The difference in egg laying following feeding on different treatments was compared using a Kruskal–Wallis test, followed

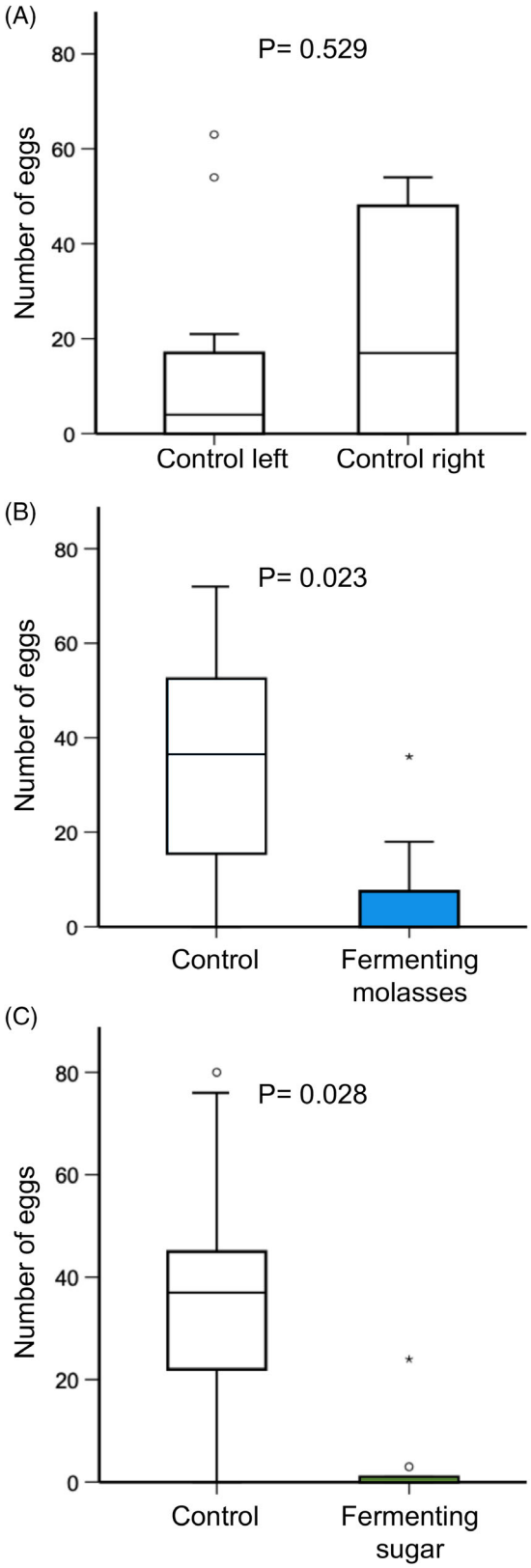

FIGURE 2 Oviposition response of mosquitoes to volatiles from fermenting molasses and sugar. Boxplots show two-choice tests with individual mosquitoes: (a) control versus control ($N = 13$), (b) control versus fermenting molasses ($N = 12$) and (c) control versus fermenting sugar ($N = 9$). *p*-Values are indicated (Wilcoxon signed rank test)

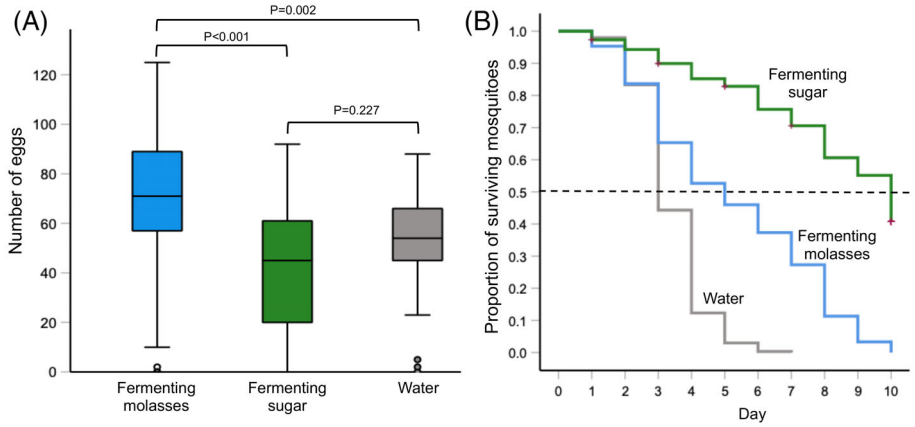

FIGURE 3 Effect of fermenting molasses and sugar on reproduction and survival. (a) Boxplot showing the effect of fermenting molasses ($N = 81$), fermenting sugar ($N = 59$) or water ($N = 48$) on the egg laying of *Anopheles coluzzii* (Kruskal–Wallis pairwise comparison). (b) Survival of mosquitoes with access to fermenting molasses, fermenting sugar or water ($N = 60$ for each treatment). All survival curves were significantly different ($p < 0.001$, Kaplan–Meier)

by post hoc pairwise comparisons with Bonferroni correction. Differences in the effect of different treatments on mosquito survival were analysed using Kaplan–Meier analysis.

The attractiveness of the synthetic blend to blood-fed mosquitoes in the olfactometer was tested with a GLM (binomial distribution, logit link function, dispersion estimated) as described previously (Verhulst et al., 2018). The 95% confidence interval derived from the GLM of the predicted proportion of mosquitoes choosing the odour sample was used to assess whether mosquito choice differed from a 50:50 distribution (Robinson et al., 2018). The effects of temperature, humidity and position of treatment in the olfactometer, and their interactions were included as parameters in the model when significant. *p*-Values below 0.05 were considered significant and GLM models were compared using the corrected Akaike's information criterion. Analyses were performed with SPSS (IBM SPSS Statistics for Windows, Version 27.0, USA).

RESULTS

Attractiveness of fermenting molasses to blood-fed mosquitoes

An increase in CO_2 production over time was detected when sugar or molasses was fermenting (LMM, $df = 29$, $p < 0.001$), indicating that differences in CO_2 production could affect the behaviour of blood-fed mosquitoes. However, no differences in CO_2 production were detected between the two methods of fermentation (LMM, $d = 29$, $p = 0.680$, Figure S2). Optimal trapping of blood-fed mosquitoes with fermenting molasses occurred 72 h after blood-feeding and was significantly higher than the number of blood-fed mosquitoes caught with fermenting sugar (Z-test, $\chi^2 = 7.109$, $p = 0.008$, Figure S3). When similar tests were performed with non-blood-fed mosquitoes, no differences between fermenting molasses and fermenting sugar were found (Z-test, $\chi^2 = 1.035$, $p = 0.215$, Figure S4).

Fermentation of molasses increased the attraction of blood-fed (72 hours post-feeding [hpf]) mosquitoes. On average, blood-fed mosquitoes were caught in higher numbers in traps baited with fermenting molasses (79 ± 11%), compared to those caught in traps without yeast (24 ± 9%) or molasses (32 ± 22%; GLM, $df = 9$, $p < 0.001$, Figure 1). Fermenting sugar traps caught a significantly lower number of mosquitoes (31 ± 7%) than fermenting molasses traps (GLM, $df = 9$, $p < 0.001$), but not lower than traps baited with the other treatments (GLM, $df = 9$, $p > 0.662$, Figure 1). Although the time after feeding was the same for all treatments, the day of testing had a significant effect on the number of mosquitoes caught, and was included as a fixed factor in the GLM ($df = 9$, $p < 0.015$).

Oviposition experiments with molasses

In two-choice experiments, significantly fewer eggs were found in oviposition cups with fermenting molasses compared to cups containing water alone (Wilcoxon signed rank test, $Z = -2.277$, $p = 0.023$, Figure 2b). Similarly, significantly fewer eggs were laid in the oviposition cups with fermenting sugar compared to the control ($Z = -2.192$, $p = 0.028$, Figure 2c).

Effect of fermenting molasses and sugar on mosquito fitness

Of the three groups of 100 blood-fed mosquitoes, which had access to the different treatments after their bloodmeal, 81% laid eggs when they had access to fermenting molasses, 59% when they had access to fermenting sugar and 44% when they were offered water only. The treatments had a significant effect on mosquito egg laying

(Kruskal–Wallis: H[2] = 33.505, $p < 0.001$, Figure 3a), and pairwise comparisons showed that access to fermenting molasses significantly increased the number of eggs laid compared to those provided with sugar or water alone ($p < 0.001$ and $p = 0.002$, respectively, Figure 3a). There was no difference between the number of eggs laid when the mosquitoes had access to fermenting sugar or water only ($p = 0.227$, Figure 3a).

Survival of mosquitoes was the longest when they had access to fermenting sugar. Around 50% of the mosquitoes survived for ≥10 days with access to fermenting sugar water, which was significantly longer than the median survival of 5 days with access to fermenting molasses ($\chi^2[1] = 240.03$, $p < 0.001$, Figure 3b). When mosquitoes only had access to water, they had a median survival of 3 days, which was significantly lower than with access to fermenting molasses or with access to fermenting sugar (both $p < 0.001$, Figure 3b).

GC–EAD and GC–MS analyses identify behaviourally active compounds in fermenting molasses

Using GC–EAD, the antennal response of blood-fed *A. coluzzii* to pooled headspace extracts of fermenting molasses and sugar were recorded. The bioactive compounds were identified as isoamylacetate, styrene, alpha-pinene, 2-ethyl-6-methyl pyrazine, 2,3,5-trimethylpyrazine, ethyl caprylate, caprolactam and decanoic acid, ethyl ester compounds (Table 1, Figure 4). These bioactive compounds were not found in the extracts of fermenting sugar, except for caprolactam (Figure S5). The release rates of the bioactive compounds were assessed in comparison with an internal standard. A synthetic blend containing the bioactive compounds, except 2-ethyl-6-methyl pyrazine, at the ratio determined by GC–MS was generated and then tested in an olfactometer for its attractiveness for blood-fed *A. coluzzii*.

Attractiveness of a synthetic blend in an olfactometer

The fermenting molasses and three of the five doses of the synthetic blend attracted significantly more blood-fed (72 hpf) mosquitoes than the control (GLM, $df = 30$, $p < 0.001$, Figure 5). The 0.3 and 0.01 concentrations of the synthetic blend were the only two treatments not different from 50:50 ratio. The percentage of mosquitoes caught in both trapping devices, as compared to all mosquitoes released (response), was highest when fermenting molasses or the two highest

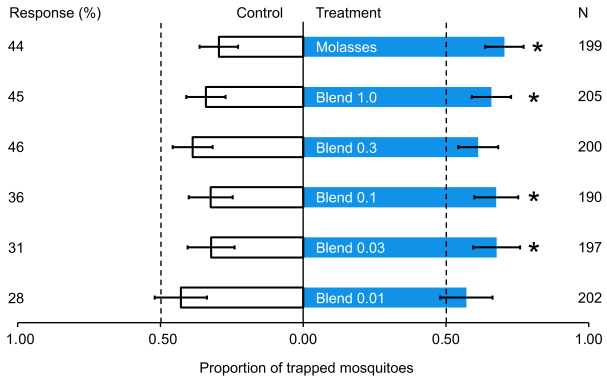

FIGURE 5 Dose dependent response of *Anopheles coluzzii* to a synthetic molasses blend 72 h after blood-feeding. The synthetic blend consisted of seven compounds at the identified ratios based on the gas chromatography–mass spectrometer analysis (see also Table 1). Fermenting molasses was included as a positive control. For each replicate, 35 blood-fed *A. coluzzii* were released. Predicted proportion of trapped mosquitoes and error bars (generalized linear model [GLM]) are presented ($n = 6$). The response is the total number of mosquitoes caught in both trapping devices as percentage from the total released. Asterisks indicate treatments that were different from a predicted 50:50 distribution, as determined by 95% two-sided confidence intervals (GLM)

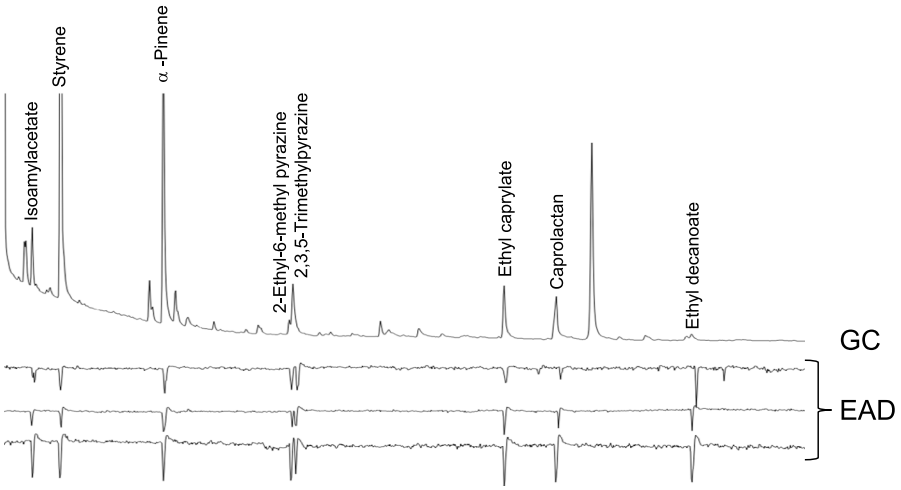

FIGURE 4 Combined gas chromatography–electroantennographic detection (GC–EAD) analysis identifies the bioactive compounds in fermenting molasses to which *Anopheles coluzzii* responds. Top: trace indicates the flame ionization detection chromatogram of the GC. Bottom: reproducible antennal responses to bioactive compounds as identified by EAD analysis (see also Table 1).

concentrations of the synthetic blend were tested, although no significant difference between treatments and control were observed (GLM, $df = 26$, $p = 0.393$, Figure 5). The response was lowest at the lowest concentration of the synthetic blend (Figure 5).

DISCUSSION

In an earlier field study, traps baited with fermenting molasses caught a high proportion of blood-fed mosquitoes (Mweresa et al., 2014). In the present study, these results were confirmed in large cage laboratory experiments, which showed that traps baited with fermenting molasses catch, on average, 79% of the blood-fed (72 hpf) mosquitoes released. Fermenting the molasses was essential to achieve this catch rate, as treatments with the yeast separated from the mixture reduced the number of blood-fed mosquitoes caught, compared to the numbers caught with fermenting sugars.

Since the attraction of fermenting molasses for mosquitoes seemed to be initiated around 2 days after the bloodmeal, we first hypothesized that fermenting molasses was an oviposition attractant, but no evidence for this was found. The results of Mweresa et al. (2014) seem to confirm this, as these authors observed an increase only in the number of blood-fed females caught with fermenting molasses, but not that of gravid females. Although fermenting molasses became more attractive as time progressed since the blood meal, it does not appear to be an oviposition attractant for *A. coluzzii*. In addition, as more eggs were laid in the control oviposition cups, fermenting molasses may even elicit a deterrent effect.

As our results indicated that fermenting molasses did not act as an oviposition attractant, we next hypothesized that fermenting molasses attracts blood-fed mosquitoes because it contains nutrients that enhance their fecundity and survival. This was, indeed, observed for fecundity (numbers of eggs laid per female), which significantly increased when fermenting molasses was used compared to sugar fermentation or water alone. However, survival was reduced when compared to fermenting sugar (Figure 3), although survival was significantly higher when compared to water only. This suggests a trade-off between fecundity and survival. An alternative hypothesis is that the ample amounts of simple carbohydrates found in the fermenting sugar support survival compared to the more complex carbohydrates and nitrogenous compounds in fermenting molasses, which support reproduction (Peters, 2006). Increased fecundity by supplementing a bloodmeal with additional nutrients has previously been observed. For example, mosquitoes may take a second bloodmeal within the same gonotrophic cycle to increase fecundity (Briegel & Hörler, 1993; Takken et al., 1998) and urea, obtained by mosquitoes after a bloodmeal from feeding on cattle urine, can also be a source of extra nutrients (Dawit et al., 2020). Cattle urine and its main nitrogenous component, urea, enhance flight activity, survival and reproductive traits, and attracts blood-fed mosquitoes, similar to our experiments with fermenting molasses.

Although CO_2 and ethanol have been hypothesized to account for the attraction of fermenting sugars, including molasses, for female mosquitoes (Mweresa et al., 2014), these are clearly not the only attractants acting in this system. Several of the bioactive volatiles identified from fermenting molasses in the GC–EAD and GC–MS analyses have previously been identified as attractants, either by themselves, or in combination with other volatile organic compounds, for various mosquito species. For example, isoamylacetate is a floral attractant for female *Aedes albopictus*, *Aedes japonicus*, *A. aegypti*, *Culex restuans* and *Culex quinquefasciatus* (Brooks, 2014), whereas alpha-pinene, is associated with human *Plasmodium sp.* infection (Emami et al., 2017; Kelly et al., 2015; Schaber et al., 2018) as well as with host plants, which in combination with other volatile organic compounds attract various physiological states of female *Anopheles* mosquitoes (Wondwosen et al., 2016, 2018, 2021). Styrene is associated with the headspace of host plants and human skin, which attract malaria mosquitoes (Pachuwah et al., 2016). Moreover, 2-ethyl-6-methyl pyrazine, isoamylacetate and ethyl decanoate have been identified in the headspace of fermenting yeasts, whereas ethyl caprylate has been identified from fermenting sugar (El-Sayed et al., 1999; Izzo & Ho, 1991; Kotseridis & Baumes, 2000; Mahadevan & Farmer, 2006; Münch et al., 1997; Smallegange et al., 2010; Stashenko et al., 1992). The studies mentioned above do not report an increase in the attraction of blood-fed individuals, emphasizing that the synthetic blend of bioactive compounds identified in this study is essential for the attraction of blood-fed mosquitoes.

The synthetic blend composed of the GC–EAD-active compounds attracted blood-fed (72 hpf) mosquitoes when tested in a dual-choice olfactometer in a dose-dependent manner. Future experiments in which individual components are subtracted from the whole synthetic blend are required to identify those that are essential for the attraction of blood-fed females. In addition, both the fermenting molasses and the synthetic blend should be tested for their attractiveness for other mosquito species (from other genera), both in laboratory and in field experiments. Attracting blood-fed mosquitoes with a synthetic blend, instead of fermenting molasses, has the advantage that it is easier to prepare and transport. Moreover, synthetic lures can be stored for longer periods, whereas the molasses mixture has to be prepared just prior to use. In addition, the 5 L plastic containers with the molasses mixture take up much more space than the vials with the synthetic blend. And finally, the molasses mixture is only effective for 24 h, whereas an optimized formulation of the synthetic blend may provide a more long-lasting lure (Mukabana et al., 2012).

A standardized synthetic blend for collecting blood-fed mosquitoes may be an additional and valuable tool for sampling blood-fed mosquitoes. For example, the human blood index is often used as an indicator of host selection of mosquitoes, but is normally determined by aspiration of mosquitoes from walls or pyrethrum spray catches indoors in which the collections are biased for human blood (Takken & Verhulst, 2013). Alternatives for the collection of blood-fed mosquitoes from less biased locations include the use of resting boxes (Brugman et al., 2017; Qiu et al., 2007), barrier screens (Burkot et al., 2013; Keven et al., 2021), gravid traps (Opoku et al., 2018) or pit shelters (Massebo et al., 2015), but the numbers of mosquitoes collected are generally low. The addition of the synthetic fermenting molasses lure could be used in combination with these collection methods to further increase the proportion of blood-fed mosquitoes collected in an unbiased manner.

Increased trap catches of blood-fed mosquitoes would also be beneficial for xenodiagnostics, whereby the bloodmeal of the insect is used to detect (non-vector borne) pathogens (Brinkmann et al., 2016). For example, analysing the blood meal of blood-engorged flies and mosquitoes demonstrated that hematophagous dipterans can act as 'flying syringes', and can be used to identify both known and unknown human pathogen species (Barbazan et al., 2008; Bitome-Essono et al., 2017). In addition to the direct identification of host pathogens in a blood meal, antibodies against pathogens can also be determined which maximizes the use of blood-fed mosquitoes to demonstrate pathogen circulation (Leighton et al., 2014; Štefanić et al., 2022).

Pathogen monitoring often involves numerous challenges, including logistical, economic and ethical issues (Cameron & Ramesh, 2021), and xenomonitoring of blood-fed mosquitoes could be an alternative method for identifying emerging human disease, when accurate and reliable blood-fed mosquito collections for screening systems have been developed. After optimization, the use of a synthetic blend to standardize blood-fed mosquito catches will allow for more accurate predictions of mosquito host selection, thereby increasing our understanding of vector-borne disease risk.

AUTHOR CONTRIBUTIONS

Sharon R. Hill, Rickard Ignell, Constantianus J. M. Koenraadt, Jeroen Spitzen and Niels O. Verhulst contributed to the conceptualization and methodology. Malou Juurlink, Betelehem Wondwosen, Sapience Rugaimukamu and Niels O. Verhulst performed the investigation and formal analysis. Malou Juurlink, Sapience Rugaimukamu, Constantianus J. M. Koenraadt, Jeroen Spitzen and Niels O. Verhulst analysed data. Niels O. Verhulst wrote the original draft. All authors reviewed and edited the manuscript and approved the final manuscript.

ACKNOWLEDGEMENTS

We thank, Frans K. M. van Aggelen, Arnout Berendsen, Karst de Boer, Luna Czarnecki, André J. Gidding, Kimmy Reijngoudt, Pieter Rouweler and Leon Westerd for mosquito rearing and Willem Takken and Alexander Mathis for their support and suggestions on the methodology. Mengistu Dawit is thanked for his help with headspace collections. Elin Isberg is thanked for her help with the wick dispensers. This work was supported by a grant from the Earth and Life Sciences Foundation (Veni-ALW) of the Netherlands Organization for Scientific Research (NWO, 863.13.012). We highly acknowledge the Swiss Federal Food Safety and Veterinary Office as sponsor of the Swiss National Centre for Vector Entomology. The graphical abstract was created with BioRender.com.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on Mendeley Data: doi: 10.17632/85zd3nf22d.1.

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REFERENCES

Barbazan, P., Thitithanyanont, A., Missé, D., Dubot, A., Bosc, P., Luangsri, N. et al. (2008) Detection of H5N1 avian influenza virus from mosquitoes collected in an infected poultry farm in Thailand. *Vector-Borne and Zoonotic Diseases*, 8, 105–110.

Birgersson, G., Dalusky, M.J., Espelie, K.E. & Berisford, C.W. (2012) Pheromone production, attraction, and interspecific inhibition among four species of *Ips* bark beetles in the southeastern USA. *Psyche*, 2012, 532652.

Bitome-Essono, P.-Y., Ollomo, B., Arnathau, C., Durand, P., Mokoudoum, N. D., Yacka-Mouele, L. et al. (2017) Tracking zoonotic pathogens using blood-sucking flies as 'flying syringes'. *eLife*, 6, e22069.

Briegel, H. & Hörler, E. (1993) Multiple blood meals as a reproductive strategy in anopheles (Diptera: Culicidae). *Journal of Medical Entomology*, 30, 975–985.

Brinkmann, A., Nitsche, A. & Kohl, C. (2016) Viral metagenomics on blood-feeding arthropods as a tool for human disease surveillance. *International Journal of Molecular Sciences*, 17, 1743.

Brooks, C. (2014) *Cage bioassay of sugar and floral source attraction in mosquitoes* (MSc thesis). University of Georgia, Athens, Georgia.

Brugman, V., Hernández-Triana, L., England, M., Medlock, J., Mertens, P., Logan, J. et al. (2017) Blood-feeding patterns of native mosquitoes and insights into their potential role as pathogen vectors in the Thames estuary region of the United Kingdom. *Parasites & Vectors*, 10, 163.

Burkot, T.R., Russell, T.L., Reimer, L.J., Bugoro, H., Beebe, N.W., Cooper, R. D. et al. (2013) Barrier screens: a method to sample blood-fed and host-seeking exophilic mosquitoes. *Malaria Journal*, 12, 49.

Cameron, M.M. & Ramesh, A. (2021) The use of molecular xenomonitoring for surveillance of mosquito-borne diseases. *Philosophical Transactions of the Royal Society B*, 376, 20190816.

Cardé, R.T. (2015) Multi-cue integration: how female mosquitoes locate a human host. *Current Biology*, 25, R793–R795.

Coutinho-Abreu, I.V., Riffell, J.A. & Akbari, O.S. (2021) Human attractive cues and mosquito host-seeking behavior. *Trends in Parasitology*, 38, 246–264.

Dawit, M., Hill, S.R., Birgersson, G., Tekie, H. & Ignell, R. (2020) Malaria mosquitoes acquire and allocate cattle urine to enhance life history traits. *Malaria Journal*, 21, 180.

El-Sayed, A., Bengtsson, M., Rauscher, S. & Löfqvist, J. (1999) Multicomponent sex pheromone in codling moth (Lepidoptera: Tortricidae). *Environmental Entomology*, 28, 775–779.

Emami, S.N., Lindberg, B.G., Hua, S., Hill, S., Mozuraitis, R., Lehmann, P. et al. (2017) A key malaria metabolite modulates vector blood seeking, feeding, and susceptibility to infection. *Science*, 355, 1076–1080.

Fauver, J.R., Gendernalik, A., Weger-Lucarelli, J., Grubaugh, N.D., Brackney, D.E., Foy, B.D. et al. (2017) The use of Xenosurveillance to detect human bacteria, parasites, and viruses in mosquito bloodmeals. *The American Journal of Tropical Medicine and Hygiene*, 97, 324–329.

Fikrig, K. & Harrington, L.C. (2021) Understanding and interpreting mosquito blood feeding studies: the case of *Aedes albopictus*. *Trends in Parasitology*, 37, 959–975.

Hiscox, A., Otieno, B., Kibet, A., Mweresa, C.K., Omusula, P., Geier, M. et al. (2014) Development and optimization of the Suna trap as a tool for mosquito monitoring and control. *Malaria Journal*, 13, 257.

Izzo, H.V. & Ho, C.T. (1991) Isolation and identification of the volatile components of an extruded autolyzed yeast extract. *Journal of Agricultural and Food Chemistry*, 39, 2245–2248.

Kelly, M., Su, C.-Y., Schaber, C., Crowley, J.R., Hsu, F.-F., Carlson, J.R. et al. (2015) Malaria parasites produce volatile mosquito attractants. *MBio*, 6, e00235-15.

Keven, J.B., Katusele, M., Vinit, R., Rodríguez-Rodríguez, D., Hetzel, M.W., Robinson, L.J. et al. (2021) Nonrandom selection and multiple blood feeding of human hosts by Anopheles vectors: implications for

malaria transmission in Papua New Guinea. *The American Journal of Tropical Medicine and Hygiene*, 105, 1747–1758.
Kotseridis, Y. & Baumes, R. (2000) Identification of impact odorants in Bordeaux red grape juice, in the commercial yeast used for its fermentation, and in the produced wine. *Journal of Agricultural and Food Chemistry*, 48, 400–406.
Kweka, E. & Mahande, A. (2009) Comparative evaluation of four mosquitoes sampling methods in rice irrigation schemes of lower Moshi, northern Tanzania. *Malaria Journal*, 8, 149.
Leighton, B.J., Roitberg, B.D., Lowenberger, C.A. & Belton, P. (2014) Host antibodies in mosquito bloodmeals: a potential tool to detect and monitor infectious diseases in wildlife. *Journal of Medical Entomology*, 45, 470–475.
Mahadevan, K. & Farmer, L. (2006) Key odor impact compounds in three yeast extract pastes. *Journal of Agricultural and Food Chemistry*, 54, 7242–7250.
Massebo, F., Balkew, M., Gebre-Michael, T. & Lindtjørn, B. (2015) Zoophagic behaviour of anopheline mosquitoes in Southwest Ethiopia: opportunity for malaria vector control. *Parasites & Vectors*, 8, 1–9.
Mukabana, W., Mweresa, C., Omusula, P., Orindi, B., Smallegange, R., van Loon, J. et al. (2012) Evaluation of low density polyethylene and nylon for delivery of synthetic mosquito attractants. *Parasites & Vectors*, 5, 202.
Münch, P., Hofmann, T. & Schieberle, P. (1997) Comparison of key odorants generated by thermal treatment of commercial and self-prepared yeast extracts: influence of the amino acid composition on odorant formation. *Journal of Agricultural and Food Chemistry*, 45, 1338–1344.
Mweresa, C.K., Omusula, P., Otieno, B., Van Loon, J.J., Takken, W. & Mukabana, W.R. (2014) Molasses as a source of carbon dioxide for attracting the malaria mosquitoes *Anopheles gambiae* and *Anopheles funestus*. *Malaria Journal*, 13, 160.
Omondi, B.A., Majeed, S. & Ignell, R. (2015) Functional development of carbon dioxide detection in the maxillary palp of *Anopheles gambiae*. *The Journal of Experimental Biology*, 218, 2482–2488.
Opoku, M., Minetti, C., Kartey-Attipoe, W.D., Otoo, S., Otchere, J., Gomes, B. et al. (2018) An assessment of mosquito collection techniques for xenomonitoring of anopheline-transmitted lymphatic Filariasis in Ghana. *Parasitology*, 145, 1783–1791.
Pachuwah, P., Jürgens, A. & Johnson, S. (2016) Using floral scent as an attractant to monitor mosquito populations. *South African Journal of Botany*, 100, 343.
Peters, D. (2006) Carbohydrates for fermentation. *Biotechnology Journal: Healthcare Nutrition Technology*, 1, 806–814.
Qiu, Y.T., Spitzen, J., Smallegange, R.C. & Knols, B. (2007) Monitoring systems for adult insect pests and disease vectors. In: Takken, W. & Knols, B. (Eds.) *Emerging pests and vector-borne diseases in Europe*. Wageningen Academic Publishers: Wageningen, pp. 329–353.
Robinson, A., Busula, A.O., Voets, M.A., Beshir, K.B., Caulfield, J.C., Powers, S.J. et al. (2018) *Plasmodium* associated changes in human odor attract mosquitoes. *Proceedings of the National Academy of Sciences*, 115, E4209–E4218.
Schaber, C.L., Katta, N., Bollinger, L.B., Mwale, M., Mlotha-Mitole, R., Trehan, I. et al. (2018) Breathprinting reveals malaria-associated biomarkers and mosquito attractants. *The Journal of Infectious Diseases*, 217, 1553–1560.
Smallegange, R.C., Schmied, W., van Roey, K., Verhulst, N.O., Spitzen, J., Mukabana, W. et al. (2010) Sugar-fermenting yeast as an organic source of carbon dioxide to attract the malaria mosquito *Anopheles gambiae*. *Malaria Journal*, 9, 292.
Stashenko, H., Macku, C. & Shibamoto, T. (1992) Monitoring volatile chemicals formed from must during yeast fermentation. *Journal of Agricultural and Food Chemistry*, 40, 2257–2259.
Štefanić, S., Grimm, F., Mathis, A., Winiger, R. & Verhulst, N.O. (2022) Xenosurveillance proof-of-principle: detection of *Toxoplasma gondii* and SARS-CoV-2 antibodies in mosquito blood meals by (pan)-specific ELISAs. *Current Research in Parasitology & Vector-Borne Diseases*, 2, 100076.
Takken, W. & Knols, B.G.J. (2010) *Olfaction in vector-host interactions*. Wageningen: Wageningen Academic Publishers.
Takken, W. & Verhulst, N.O. (2013) Host preferences of blood-feeding mosquitoes. *Annual Review of Entomology*, 58, 433–453.
Takken, W., Klowden, M.J. & Chambers, G.M. (1998) Effect of body size on host seeking and blood meal utilization in *Anopheles gambiae* sensu stricto (Diptera: Culicidae): the disadvantage of being small. *Journal of Medical Entomology*, 35, 639–645.
Verhulst, N.O., Loonen, J.A.C.M. & Takken, W. (2013) Advances in methods for colour marking of mosquitoes. *Parasites & Vectors*, 6, 200.
Verhulst, N.O., Umanets, A., Weldegergis, B.T., Maas, J.P.A., Visser, T.M., Dicke, M. et al. (2018) Do apes smell like humans? The role of skin bacteria and volatiles of primates in mosquito host selection. *Journal of Experimental Biology*, 221, jeb.185959.
Wolff, G.H. & Riffell, J.A. (2018) Olfaction, experience and neural mechanisms underlying mosquito host preference. *Journal of Experimental Biology*, 221, jeb157131.
Wondwosen, B., Birgersson, G., Seyoum, E., Tekie, H., Torto, B., Fillinger, U. et al. (2016) Rice volatiles lure gravid malaria mosquitoes, *Anopheles arabiensis*. *Scientific Reports*, 6, 1–8.
Wondwosen, B., Birgersson, G., Tekie, H., Torto, B., Ignell, R. & Hill, S. R. (2018) Sweet attraction: sugarcane pollen-associated volatiles attract gravid *Anopheles arabiensis*. *Malaria Journal*, 17, 1–9.
Wondwosen, B., Dawit, M., Debebe, Y., Tekie, H., Hill, S.R. & Ignell, R. (2021) Development of a chimeric odour blend for attracting gravid malaria vectors. *Malaria Journal*, 20, 1–9.
Wooding, M., Naudé, Y., Rohwer, E. & Bouwer, M. (2020) Controlling mosquitoes with semiochemicals: a review. *Parasites & Vectors*, 13, 80.
Yan, J., Gangoso, L., Ruiz, S., Soriguer, R., Figuerola, J. & Martínez-de la Puente, J. (2021) Understanding host utilization by mosquitoes: determinants, challenges and future directions. *Biological Reviews*, 96, 1367–1385.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Supplementary Figure 1. Cage design of the oviposition experiment and the oviposition cup (top right and bottom).

Supplementary Figure 2. Boxplot of the amount of carbon dioxide produced by fermenting molasses or fermenting sugar.

Supplementary Figure 3: Percentage of blood-fed mosquitoes trapped inside the BG-Suna trap baited with either fermented molasses of sugar after release at different time points post-blood-feeding.

Supplementary Figure 4: Percentage non-blood-fed mosquitoes trapped inside the BG-Suna trap baited with either fermented molasses of sugar after release at different time points.

Supplementary Figure 5. Combined gas chromatography–electroantennographic detection analysis identifies the bioactive compounds in fermenting molasses to which *Anopheles coluzzii* responds.

How to cite this article: Verhulst, N.O., Juurlink, M., Wondwosen, B., Rugaimukamu, S., Hill, S.R., Ignell, R. et al. (2022) Fermenting molasses and a synthetic odour blend to attract blood-fed *Anopheles coluzzii*. *Medical and Veterinary Entomology*, 1–10. Available from: https://doi.org/10.1111/mve.12622