Floral Odors and the Interaction Between Pollinating Ceratopogonid Midges and Cacao

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Abstract - Most plant species depend upon insect pollination services, including many cash and subsistence crops. Plants compete to attract those insects using visual cues and floral odor which pollinators associate with a reward. The cacao tree, *Theobroma cacao*, has a highly specialized floral morphology permitting pollination primarily by Ceratopogonid midges. However, these insects do not depend upon cacao flowers for their life cycle, and can use other sugar sources. To understand how floral cues mediate pollination in cacao we developed a method for rearing Ceratopogonidae through several complete lifecycles to provide material for bioassays. We carried out collection and analysis of cacao floral volatiles, and identified a bouquet made up exclusively of saturated and unsaturated, straight-chain hydrocarbons, which is unusual among floral odors. The most abundant components were tridecane, pentadecane, (*Z*)-7-pentadecene and (*Z*)-8-heptadecene with a heptadecadiene and heptadecatriene as minor components. We presented adult midges, *Forcipomyia* sp. (subgen*. Forcipomyia*)*, Culicoides paraensis* and *Dasyhelea borgmeieri,* with natural and synthetic cacao flower odors in choice assays. Midges showed weak attraction to the complete natural floral odor in the assay, with no significant evidence of interspecific differences. This suggests that cacao floral volatiles play a role in pollinator behavior. Midges were not attracted to a synthetic blend of the above four major components of cacao flower odor, indicating that a more complete blend is required for attraction. Our findings indicate that cacao pollination is likely facilitated by the volatile blend released by flowers, and that the system involves a generalized odor response common to different species of Ceratopogonidae.

Key words - Floral traits; Flower odor; Cacao; Ceratopogonidae; Cocoa midges; Tropical agriculture; Behavioral ecology; (*Z*)-7-Pentadecene; (*Z*)-8-Heptadecene

Introduction

A variety of cues can be used by pollinators to locate flowers, including color (Arnold et al. 2010), shape (Dafni et al. 1997), pattern (Van Kleunen et al. 2007), temperature (Dyer et al. 2006), and odor (Raguso 2008; Schiestl 2010). Floral characteristics such as odor can evolve in order to favor more efficient pollinators over less efficient ones. For example, *Mimulus lewisii* Pursh emits D-limonene, β-myrcene, and (*E*)-β-ocimene, a blend which attracts bumblebees preferentially. Conversely, its sister species *M. cardinalis* Douglas ex. Benth emits very low levels of these compounds and receives few bumblebee visits, but is readily pollinated by hummingbirds (Byers et al. 2014a; Byers et al. 2014b). The odor blend thus serves as a selective filter to pollinators, increasing pollination efficacy.

One flower with a specialized morphology is that produced by the cacao tree, *Theobroma cacao* L. (Malvaceae). The small flowers have the anthers concealed within tiny, cup-shaped "petal hoods", making the pollen inaccessible to larger pollinators, while the pistil is surrounded by long, usually dark red, staminodes (Supplementary Material Fig. S1). Compared to many crops such as temperate soft- and top-fruit, the plant-pollinator interactions in this crop species are relatively poorly understood. This lack of knowledge persists despite cacao's global importance that stems from its widespread cultivation across the tropics as the source of cocoa. Pollination rates in cacao are often low (Forbes and Northfield 2017; Groeneveld et al. 2010) and yields are poor in many countries.

The insect pollinators of cultivated varieties of cacao are generally thought to be midges in the family Ceratopogonidae (Ceratopogonidae previously recorded visiting cacao flowers will henceforth be termed "cocoa midges" for ease), with different species performing this service in different regions (Winder 1978a). The reasons for visiting the flowers remain unclear, but access to flowers increases female longevity (Saunders 1959). Wherever cacao has been introduced, native midge species are recorded visiting the flowers, and where inspected, transferring pollen (O'Doherty and Zoll 2012). In locations where multiple midge species are present, e.g. Ghana (Kaufmann 1975), the Caribbean (Arnold et al. 2018), Costa Rica, and Brazil (Winder 1977), evidence is often limited as to which are the most efficient pollinators. A secondary pollination service may be contributed by other small Diptera (Kaufmann 1973; Winder 1978b), but there is less evidence of the

importance of non-ceratopogonid flies on most plantations. Recent studies of wild trees also indicate high levels of visitation by small Hymenoptera, such as chalcid wasps (Chumacero de Schawe et al. 2018), but there is little evidence of this occurring to a large extent in cultivated systems (Frimpong et al. 2009; Winder 1978a). The midge-cacao pollination system is of particular interest because different life stages of Ceratopogonidae can make use of different parts of the same cacao plant: adults benefit from visits to the flowers, while larvae can develop in discarded rotting cacao pods (Winder 1978a). Nonetheless, with the exception of some limited studies (Brew 1988; Erickson et al. 1987; Young et al. 1989), there remains a lack of knowledge about the mechanisms mediating the midge-flower interactions, particularly the cues inducing midges to land on and enter flowers.

Only a small number of studies have examined the odor bouquet of cacao flowers. In studies from Costa Rica, Erickson et al. (1987) and Young and Severson (1994) reported 1-pentadecene, *n*-pentadecane and 1-heptadecene as major components. However, the authors were unable to demonstrate that these compounds mediated any behavioral responses of the main pollinating taxon (Diptera: Ceratopogonidae) in the field (Young et al. 1989). Cacao-visiting midges have proven very difficult to rear in the laboratory across multiple generations (Saunders 1959), making controlled behavioral studies difficult or impossible.

In this study we describe the volatile compounds sampled from flowers of cacao plants from farms in the Caribbean. We report a methodology that resulted in reliable emergence of ceratopogonid midges for several months in a laboratory environment, providing sufficient adults for bioassays. We describe the results of controlled choice-tests in a Y-tube olfactometer using cocoa midges, in which adult females were allowed to choose between control odors or natural and synthetic odors of cacao flowers to test whether cacao floral volatiles are attractive to the midges and whether a synthetic blend could elicit comparable behaviors.

Methods and Materials

Sampling Floral Odors

Odors were sampled from cacao trees of Imperial Mixed Calabacillo (IMC) and Trinidad Selected Hybrid (TSH) cultivars. The trees were located on farms in St Mary Parish, Jamaica (18° 13′ 59″ N 76° 52′ 55″ W), and Gran Couva (10° 25′ 17″ N 61° 20′ 8″ W) and La Réunion (10° 35′ 30″ N 61° 18′ 15″W), Trinidad and Tobago (Arnold et al. 2018). Sampling took place in Trinidad in 2012 and Jamaica in 2013, providing samples from both the wet and dry seasons (Supplementary Material Table S1).

Cacao trees are cauliflorous with flowers emerging from flower cushions directly on the trunk and branches. Accessible branches or trunk sections with 1-4 open, fresh flowers were selected for sampling, and comparable sections without flowers were also sampled. These sections, approximately 20 cm long, were enclosed in a poly(ethyleneterephthalate) oven bag (37 x 25 cm x 12 µm thick; J Sainsbury plc, UK) (Stewart-Jones and Poppy 2006). The oven bags had been previously tested for volatile emissions and determined to be odorless. Two battery-powered pumps (NMP 830 KNDC-B; KNF Neuberger, Freiburg, Germany) were used, one to pump charcoal-filtered air through Teflon tubing (1.6 mm i.d. x 3.2 mm o.d.) into the bag (600 cm^3 min^{-1}) and the other to draw air out of the bag (500 cm^3 min^{-1}) through a collection filter, thus maintaining a positive pressure in the bag to avoid introduction of impurities. Collection filters consisted of a Pasteur pipette (4 mm i.d.) containing Porapak Q (200 mg, 50–80 mesh; Supelco, Gillingham, Dorset, UK) held between plugs of silanized glass wool. The Porapak Q was purified by Soxhlet extraction with dichloromethane (Pesticide Residue Grade, Fisher Scientific, Loughborough, UK) for 8 h and washing with dichloromethane before use. Cacao flowers ordinarily commence anthesis in late afternoon/evening, are fully open from early in the morning the following day, and remain receptive into the afternoon (Sampayan 1966). They senesce over the following 24-48 h (Aneja et al. 1999). Volatiles were collected for 24 h, starting at 09:00-12:00, after which collection filters were wrapped in aluminum foil and transported to the Natural Resources Institute (NRI), Chatham Maritime, UK. Volatiles were eluted with dichloromethane (1 ml) and samples stored at -20 °C until analysis.

Chemical Analyses

Samples were initially analyzed on an Agilent 6890 gas chromatograph (GC) coupled to an Agilent 5973 mass spectrometer (MS) (Agilent Technologies, Manchester, UK) fitted with a fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) coated with DB-5 (Agilent). Carrier gas was helium (1 ml min^{-1}), injection was splitless (220 °C) and the oven temperature was held at 50 °C for 2 min, then heated to 250 °C at 6 °C min^{-1} and held for 5 min. The ion source was held at 230 °C, and the transfer line was at 250 °C.

Compound identifications were confirmed by analyses on a polar GC column using a Varian CP-3800 GC coupled directly to a Saturn 2200 MS (Varian, now Agilent). This was fitted with two fused silica capillary columns (30 m x 0.25 mm i.d. x 0.25 µm film thickness) coated with polar DBWax (Agilent) and non-polar VF5 (Varian) respectively, and a column switching device. Carrier gas was helium (1 ml min^{-1}), injection was splitless (220 °C and 250 °C respectively) and oven temperature was held at 40 °C for 2 min then programmed at 10 °C min^{1} to 250 °C and held for 5 min.

Retention indices of compounds were calculated from their retention times relative to those of *n*-alkanes analyzed under the same conditions. Compounds were identified by comparison of their mass spectra and retention indices relative to those of authentic synthesized standards on both columns.

Positions of double bonds in unsaturated compounds were determined by GC-MS analyses of their dimethyl disulfide (DMDS) derivatives (Buser et al., 1983; Carlson et al., 1989). An aliquot (100 µl) of a collection of volatiles estimated to contain approximately 1 µg of the major alkene was evaporated just to dryness under a gentle stream of nitrogen and then treated with dimethyl disulfide (10 µl; SigmaAldrich, Gillingham, Dorset, UK) and a 5% solution of iodine in diethyl ether (10 µl) in a sealed vial. After heating at 40 °C for 4 h the mixture was dissolved in hexane (100 µl) and extracted twice with 5% aqueous sodium thiosulfate solution (100 µl) before analysis by GC-MS on the VF5 column above.

Synthetic Compounds

Tridecane, tetradecane, pentadecane, hexadecane, heptadecane and 1-pentadecene were purchased from SigmaAldrich.

(*Z*)-7-Pentadecene and (*Z*)-8-heptadecene were synthesized by Wittig reaction between octyl(triphenylphosphonium) bromide and freshly-distilled heptanal or nonanal, respectively, with potassium *t*-butoxide in tetrahydrofuran at 0 °C. Products were purified by flash chromatography on silica gel in hexane followed by kugelrohr distillation, and were 98% chemically pure (95% isomerically pure, with approximately 5% of the (*E*)-isomers).

(*Z,Z*)-6,9-Heptadecadiene and (*Z,Z,Z*)-3,6,9-heptadecatriene were synthesized by decarboxylation of linoleic acid ((*Z,Z*)-9,12-octadecadienoic acid) and linolenic acid ((*Z,Z,Z*)-9,12,15-octadecatrienoic acid) respectively, and characterized according to van der Klis et al. (2011). Yields were low (approx 10%), as reported by Klis et al. (2011), but purities were remarkably high (> 97% by GC analysis) after flash chromatography and kugelrohr distillation.

Collection of Cocoa Midges

Ceratopogonid cocoa midges were obtained from detritus materials (rotting cacao pods, banana pseudostem) collected from a plantation in Gran Couva, Trinidad, (10° 25’ 17” N, 61° 20’ 8” W). The detritus was brought to an insectary facility at the University of Trinidad and Tobago and placed in emergence cages (*N* = 10, 475 x 475 mm Bugdorm, Taichung, Taiwan) under ambient conditions. The cages were misted with 10% sucrose solution 3-4 times per week and slices of organic apple were provided for moisture and sugar.

To encourage pupal eclosion prior to midge collection (approximately 6 weeks after the commencement of rearing), the detritus was heavily wetted with 250 ml distilled water per cage, resulting in high pupal eclosion rates approximately two days later. Collections were conducted using a mouth-operated aspirator (pooter) to transfer midges into collection tubes lined with dampened filter paper (10% sucrose solution in water) and sealed with cheesecloth material to permit air diffusion. In total, around 500 adult

Ceratopogonidae were collected over two days. Larvae and pupae were also observed in the detritus by visual inspection, collected using fine forceps and transferred into collection vials, also totaling around 500 individuals between 10 vials. Each vial was provisioned with a "cacao substrate ball", made from rotting cacao husk rolled into a 10-15 g ball, to provide food and habitat for developing larvae during transit. The adults, larvae and around 250 pupae were then transported to NRI, UK, in October 2015. A second collection of larvae and pupae was made in March 2016 and added to the culture to boost populations.

Upon arrival in the UK, midges and detritus materials were transferred to smaller cages (300 x 300 x 300 mm) in a controlled temperature room (26 °C and 60% RH and a 14:10 L:D cycle). Each cage was placed completely within a large transparent polythene bag to retain moisture, which was secured with an elastic band. High mortality of adults was observed in the 1-2 days following transit, but larvae/pupae survived transit better. Maintenance proceeded as previously, and the cages were provided with damp leaf litter and rotten pumpkin as potential breeding sites.

The three main species in this experiment were *Dasyhelea borgmeieri* Wirth, *Culicoides paraenesis* Goeldi and *Forcipomyia* sp. Meigen. Specimens of all three species were identified at the Museo de La Plata by a Ceratopogonidae specialist (GRS) using morphological species descriptions and taxonomic keys for neotropical Ceratopogonidae.

Cacao pod could not be obtained in the UK, but the three main species in this experiment have been previously reported from a variety of decomposing vegetable material including leaf litter and plant detritus (Winder 1978a). The walls of the cage and the inside of the polythene bag were inspected daily to monitor for emergence.

Olfactometry

The olfactometry study aimed to characterize the responses of cocoa midges to both natural cacao flower odors and a synthetic blend. Individuals were used only once for an experiment. While the insects were kept in mixed-sex cages, males were infrequently recorded and since individuals were typically tested soon after emergence, it is possible that not all the females were mated. However, as rearing was difficult and little is known about

the mating ecology of the species tested, it was not feasible to eclose adults singly and force mating before the tests. Nevertheless, unmated and mated females both need to sugar-feed and therefore were expected to exhibit similar behaviors in response to floral odors.

Sex was determined by inspection of antennae using a hand lens: male Ceratopogonidae have plumed antennae resembling a fine paintbrush and females have simple antennae with short hairs only (Borkent and Spinelli 2007). As females are reported to be the major pollinators (Winder 1978a), the availability of predominantly females was considered not to affect the ecological relevance of this study. In total, 156 female ceratopogonid individuals were trialed. We elected not to test male individuals because very few were observed in culture cages. Individual adult females of *Dasyhelea borgmeieri* Wirth (throughout) and *Culicoides paraenesis* Goeldi/*Forcipomyia* sp. Meigen (during the first month) were thus collected within a week of emergence for trials by capturing them inside a pipette tip (Supplementary Material Fig. S2). In total, 66 females of three different species were tested for their preference between natural odor or solvent control: four of *Forcipomyia* sp., 19 of *C. paraensis* and 43 of *D. borgmeieri*. A further 90 females of *D. borgmeieri* only (as this species was most numerous in the culture) were trialed against synthetic odors versus solvent control, of which 44 were tested using the natural equivalent concentration (synthetic cacao floral odour, "SCFO 100%") and 46 at 10% of this concentration ("SCFO 10%").

Individuals were tested singly in a glass Y-tube olfactometer (Supplementary Material Fig. S3) (arm length 70 mm, angle between the arms 120°, 8 mm i.d.). Air was pushed by a pump (FB65540, Fisher Scientific) through a charcoal filter (1) to remove volatile contaminants. The airstream was then split (2) and passed through two gas-wash bottles (3), one containing the stimulus and the other the solvent control, and then into each of the two arms of the olfactometer (4), at a flow rate of 100 cm^3 min^{-1} through each arm. The two arms met and merged (5) ("decision point") into the third arm of the apparatus (6) ("approach arm"). Components were connected with Tygon tubing (internal i.d. 8 or 6 mm; Saint-Gobain, Paris, France). A lamp positioned centrally between the two arms illuminated the whole setup as Ceratopogonidae are strongly positively phototactic (Blackwell et al. 1994). The entire apparatus was shrouded by black plastic on three sides and the top to minimize visual distractions.

Individual insects were introduced into the approach arm of the olfactometer using an adapted pipette tip plugged with cotton wool; they left this readily and proceeded towards the decision point. The approach arm was then covered with dark plastic to discourage the midges from returning to the start point and leaving the apparatus. A decision was recorded when the insect entered one or the other arm of the olfactometer, and the time taken by the insect from the pipette tip to decision point was also noted. If the insect did not make a decision within 10 min, the trial was terminated and the insect excluded from analysis.

Tests were carried out between 09:00 and 12:00, as preliminary experiments indicated that midges tested after 12:00 were less likely to make a decision within the 10 min observation period. Observations of ceratopogonid midges associated with cacao flowers in the field have, similarly, observed an activity peak in the morning (Frimpong et al. 2009). Tests were performed at 26 °C and 60% RH.

For bioassays, the collection of cacao flower volatiles containing most material was used. This was estimated to contain 10 ng μl^{-1} of the major component, (*Z*)-7-pentadecene, by GC analysis in comparison with analyses of known concentrations of the synthetic compound. For the synthetic cacao flower odor (SCFO), a blend of the four major components of cacao floral odor was prepared, containing tridecane, pentadecane, (*Z*)-7-pentadecene and (*Z*)-8-heptadecene in 0.5 : 1 : 1 : 0.5 ratio, in dichloromethane like the collection of natural flower volatiles. This was tested at two concentrations, one with the (*Z*)-7-pentadecene at 10 ng μl^{-1} (SCFO 100%), making it equivalent to natural odor intensity, and after the second after dilution x 10 (SCFO 10%).

For each test, an odor solution or a dichloromethane blank (solvent control) (10 µl) was pipetted onto a 20 mm diameter filter paper in the gas-wash bottle (preliminary experiments with 1 µl of stimulus did not provoke a response). After every fifth trial, odor stimuli were replaced, and stimuli and control bottle positions were switched. After every 8-10 individuals, the Y-tube apparatus was flushed through with industrial methylated spirits (IMS; denatured ethanol) and allowed to air-dry to remove any odor cues from previous midges.

Statistics

For each floral odor stimulus (natural floral odor, SCFO 100%, SCFO 10%) tested in the olfactometer assays, differences in the number of midges choosing stimulus versus control were analyzed for significance using a binomial test. X^2 tests were used to determine whether there were differences among the three genera of Ceratopogonidae in their preference for the odor stimulus over control, when tested against natural cacao floral odor. A partial correlation, controlling for the identity of the odor blend, was also performed, to evaluate whether there was a within-day effect on midges' preferences. The correlation evaluated the relationship between the number of trials since the last cleaning of the Y-tube ("trial stage"), and the probability of a midge at a given trial stage choosing the odor. All tests were performed in SPSS version 23 (IBM Corp., NY, USA).

Results

Analysis of Floral Volatiles.

During 2012, volatiles were collected from three samples with flowers and one with bark only. In 2013, collections were made from 10 flower samples and eight with bark only. Collections were analyzed by GC-MS and only those made from cacao flowers contained reliably quantifiable amounts of volatile compounds. These were identified as a series of saturated and unsaturated, straight-chain hydrocarbons (Fig. 1, Table 1; Supplementary Material Fig. S4, S5). The main components were tridecane, pentadecane, a mono-unsaturated 15-carbon and a mono-unsaturated 17-carbon hydrocarbon. Mono-unsaturated 13-carbon, 14-carbon and 16-carbon hydrocarbons were also detected. The mono-unsaturated hydrocarbons were characterized by their GC retention indices (Table 1) and their mass spectra, showing small molecular ions (*m/z* 210 and 238 for the 15- and 17-carbon compounds, respectively), and major ions at *m/z* 138, 125, 111, 97, 83, 69, 55 (Supplementary Material Fig. S6). Traces of di-unsaturated 16-carbon and 17-carbon hydrocarbons (molecular ions at *m/z* 222 and 236, respectively, other ions at m/z 138, 123, 109, 95, 81, 67 (base peak); Supplementary Material Fig. S7) were detected, as was a tri-

unsaturated 17-carbon hydrocarbon (molecular ion at *m/z* 234, other ions at *m/z* 135, 121, 108, 93, 79 (base peak), 67; Supplementary Material Fig. S7).

GC-MS analysis of the derivatives resulting from the reaction of a representative collection of volatiles with dimethyldisulfide (DMDS) showed the two major mono-unsaturated compounds were 7-pentadecene and 8-heptadecene respectively (Table 1, Supplementary Material Fig. S8). Comparison of retention indices with those of synthetic standards on both non-polar and polar GC columns confirmed that they were mainly the (*Z*)-isomers, but approximately 5% of the corresponding (*E*)-isomers were also present, as evident in analyses of both underivatized and derivatized samples (Table 1, Supplementary Material Fig. S4).

The above GC-MS analyses of the collection of cacao flower volatiles treated with DMDS showed derivatives with GC retention times and mass spectra (Table 1) indicating that the 13-carbon mono-unsaturated hydrocarbon was the 5-isomer, the 14-carbon homologue was a mixture of approximately equal quantities of the 6- and 7-isomers, and the 16-carbon homologue was a mixture of the 7- and 8-isomers.

1-Pentadecene was reported to be the major component of volatiles from cacao flowers by Erickson et al. (1987) and Young and Severson (1994). This compound had a similar mass spectrum to that of the major, mono-unsaturated 15-carbon hydrocarbon present in our volatile collections (Supplementary Material Fig. S6), but it had clearly different GC retention indices on both non-polar and polar GC columns (1495 on DB5 and 1546 on DBWax) and the DMDS derivative had a different retention index (2288 on VF5) and fragmentation pattern (*m/z* 243, 304) (Table 1, Supplementary Material Fig. S8). 1-Pentadecene could not be detected in any of the collections of volatiles (< 0.1% of (*Z*)-7-pentadecene).

The di-unsaturated, 17-carbon hydrocarbon had identical retention times on both non-polar and polar GC columns (Table 1) and mass spectrum (Supplementary Material Fig. S7) to that of authentic (*Z,Z*)-6,9-heptadecadiene. Although other isomers were not available for comparison, the di-unsaturated, 17-carbon compound in the cacao flower volatiles is proposed to be (*Z,Z*)-6,9-heptadecadiene. The di-unsaturated 16-carbon compound is thus inferred to be (*Z,Z*)-6,9-hexadecadiene.

Similarly, the tri-unsaturated 17-carbon hydrocarbon had identical retention times on both non-polar and polar GC columns (Table 1) and mass spectrum (Supplementary Material Fig. S7) to that of authentic (*Z,Z,Z*)-3,6,9-heptadecatriene. Although other isomers were not available for comparison, the tri-unsaturated, 17-carbon compound in the cacao flower volatiles is proposed to be (*Z,Z,Z*)-3,6,9-heptadecatriene.

Midge Rearing and Olfactometry

Three Ceratopogonidae species were recorded emerging from detritus: *Dasyhelea borgmeieri*, *Forcipomyia* (undetermined species of subgenus *Forcipomyia*) and *Culicoides paraensis* (Supplementary Material Fig. S2). Adults of *D. borgmeieri* continued to emerge intermittently until May 2016 (6 months after initial transport to UK), with emergence peaks indicating a generation time of around one month.

In the Y-tube bioassays there were no significant differences among the three species in the proportion of individuals choosing the natural cacao odor over the control (26/43, 14/19, 3/4 for *D. borgmeieri*, *C. paraensis*, *F. sp.* respectively; chi square test, X^2 = 1.20, *df* = 2, *P* = 0.550). Overall, significantly more midges across the three species pooled (43/66; 65.1%) chose natural cacao flower odor over the solvent control (binomial, *N* = 66, *P* = 0.019) (Fig. 2). Individually, *C. paraensis* significantly preferred the natural odour blend to the control (binomial, N = 19, P = 0.0033), whereas *D. borgmeieri* showed a non-significant preference overall (binomial, N = 19, P = 0.111), but with a generation-dependent pattern (Supplementary Material Fig. S9). Conversely, the number of *D. borgmeieri* choosing the synthetic blend of cacao floral odors compared to the solvent control did not differ significantly and did not change with time: 38.6% (17/44) chose the SCFO 100% odor blend (binomial, *N* = 44, *P* = 0.174) and 47.8% (22/46) chose the SCFO blend at 10% concentration (binomial, *N* = 46, *P* = 0.883) (Fig. 2). There was no clear or significant effect of number of previous trials since last washing of Y-tube on the choice behavior (Supplementary Material Fig. S10) (partial correlation controlling for odor blend, *P* = 0.427).

Discussion

The volatiles from cacao flowers can, alongside other stimuli, play an important role in mediating attraction of flower visitors and subsequently pollination. We sought to explore the interaction between the odor of cacao flowers and some of the insects that pollinate them. We reared ceratopogonid midges over multiple generations, and, by testing them in a binary choice assay in Y-tube olfactometer, determined that there was a weak positive response to the natural odor of cacao flowers in some species (particularly *C. paraenesis*). Our data indicate that the odors may be generally attractive to wild-caught and first-generation midges, although responses were lost in later generations of midges; even though the numbers of *Forcipomyia* sp. we were able to test were very low, 4 out of 6 selected the odor over the control. We did not observe any attraction to a partial synthetic blend we created to replicate this odor. This suggests that in the field the odor of the cacao flower will play a role in enabling flower-visiting midges to locate flowers and thus facilitate pollination. All three species we tested (*D. borgmeieri*, *C. paraensis*, *Forcipomyia* sp.), are known cacao flower visitors (Winder 1977; Young 1986) and so their behavior has implications in cocoa production.

Volatile compounds collected from cacao flowers in this study were shown to consist of a suite of saturated and unsaturated hydrocarbons, as reported by Erickson et al. (1987) and Young and Severson (1994). These authors identified the major components as 1-pentadecene and 1-heptadecene, whereas we identified those in our samples conclusively as (*Z*)-7-pentadecene and the homologous (*Z*)-8-heptadecene. The 1-isomers could not be detected. Erickson et al. (1987) obtained samples by steam distillation of cut flowers from another Trinitario variety, whereas our samples were collected by direct aeration of flowers on the stem. It is possible that the resulting samples obtained by Erickson et al. (1987) were different from ours, but they reported identification of compounds only by comparison of their mass spectra with those in the mass spectra database, and this is insufficient evidence to ascertain the position of unsaturation. It thus seems possible that the compounds were misidentified and the errors were promulgated in the subsequent paper by Young and Severson (1994).

Other significant components in the volatile collections were the saturated *n*-alkanes tridecane, tetradecane and pentadecane. Minor components included mono-unsaturated 5-tridecene, a homologue of the two major monoenes, and 6- and 7-tetradecenes and 8- and 7-hexadecenes. In the absence of the synthetic compounds, it was not possible to assign the configurations of the minor, mono-unsaturated components of cacao flower volatiles, but it is likely to be (*Z*) in comparison with that of the major mono-unsaturated compounds.

The major components, (*Z*)-7-pentadecene and (*Z*)-8-heptadecene, are probably derived biosynthetically by decarboxylation of palmitoleic acid ((*Z*)-9-hexadecenoic acid) and oleic acid ((*Z*)-9-octadecenoic acid), respectively, a previously reported plant biosynthetic pathway (Jurenka 2004). The other mono-unsaturated hydrocarbons could be derived biosynthetically by similar decarboxylations.

Other minor components were a di-unsaturated 16-carbon and a di-unsaturated 17-carbon atom compound. The latter had GC retention indices and mass spectrum consistent with it being (*Z,Z*)-6,9-heptadecadiene, and the 16-carbon homolog is thus probably (Z,Z)-6,9-hexadecadiene. These could be derived biosynthetically by successive decarboxylations of linoleic acid ((*Z,Z*)-9,12-octadecadienoic acid). A tri-unsaturated 17-carbon compound was also detected with identical GC retention indices and mass spectrum to those of (*Z,Z,Z*)-3,6,9-heptadecatriene, which could be derived biosynthetically by decarboxylation of linolenic acid ((*Z,Z,Z*)-9,12,15-octadecatrienoic acid) via known plant biosynthetic pathways.

(*Z*)-8-Heptadecene was reported as a component of the fragrance of flowers of orchids of the *Stanhopea* genus (Asparagales: Orchidaceae) by Whitten and Williams (1992), where it was accompanied by saturated hydrocarbons as well as a wide range of different terpenoid and phenylpropanoid compounds. A blend of hydrocarbons and terpenoids was also identified in volatiles collected from flowers of *Yucca glauca* (Asparagales: Asparagaceae) by Svensson et al. (2011) and, more recently, Tröger et al. (2019) reported flowers of *Y. reverchonii* produced a blend of saturated and unsaturated hydrocarbons only. This blend was similar to that reported here from cacao flowers with the major component identified as (*Z*)-8-heptadecene, accompanied by (*Z,Z*)-6,9-heptadecadiene and heptadecane as minor components (Tröger et al. 2019). These authors admitted that they had wrongly identified the heptadecene as 1-heptadecene in their earlier paper (Svensson et al. 2011).

Our synthetic blend consisted of the major components tridecane, pentadecane, (*Z*)-7-pentadecene and (*Z*)-8-heptadecene. We tested it at two different concentrations, but neither mediated attraction behavior from the midges. This indicates that the major components alone are not sufficient for eliciting the directed movement towards an odor source that we observed from the natural odor blend. It is likely that minor components are necessary for flower recognition. This has been reported in a few other study systems of pollinator-plant interactions, especially with specialist and non-bee pollinators such as fig wasps (Chen and Song 2008) or the role of DMDS and dimethyl trisulfide (DMTS) as part of an overall more complex blend in mediating attraction by flies versus wasps in African *Eucomis* species (Shuttleworth and Johnson 2010). We anticipate that the dienes and triene detected in the cacao floral odor blends could be important, either singly or in combination with major components, in facilitating midge attraction. These were not available for testing in this study, but dienes are known to mediate behavior in other Diptera such as *Drosophila melanogaster* Meigen, where 7,11-dienes mediate mating behavior (Marcillac and Ferveur 2004).

The odor composition of cacao flowers contains compounds more typical of insect cuticle, waxes and pheromones (Blomquist and Jackson 1979). (*Z*)-7-pentadecene was isolated from ant Dufour glands, along with tridecane, tetradecane, pentadecane, and heptadecanes and heptadecenes (Billen et al. 1986), and (*Z*)-8-heptadecene and analogous saturated hydrocarbons were found in the anal secretions of *Holothrips* species (Suzuki et al. 2004). This suggests a possible connection to other aspects of midge's life history. The relationship between cocoa midges and cacao is of particular note because they interact with their plant in different ways at different life-stages, the larvae developing in the rotting pods of the tree and the adults feeding in the flowers. However, adult females of many Ceratopogonidae genera (including *Forcipomyia* and *Culicoides*) also feed on blood or insect haemolymph. Volatiles associated with insects (cuticle, frass or pheromones) often function as kairomones for parasitoids and ectoparasites (Steidle et al. 2003). As the components of cacao floral odor resemble many insect kairomones, the blend may exploit pre-existing biases in the olfactory systems of midges related to host-feeding. The key volatiles may also have biological relevance for Ceratopogonidae, in conspecific interactions; as oviposition cues, e.g. those associated with certain bacterial biofilms on which midge larvae feed

(Besemer and Soria 1978; Saunders 1959); or indicating other sources of nutrition for adult female midges. However, there remains insufficient data either about cacao pollination in natural habitats, or about cocoa midge biology and behavior, to conclude anything with certainty. Electroantennography (EAG) was attempted with *D. borgmeieri* and *Forcipoymia* sp. in order to further explore their test antennal responses to the different odor components, but it was not possible to obtain reliable results due to their small size and presence of moving mouthparts.

The study provides several important new advances. Firstly, we demonstrate that rearing cocoa midge potential pollinators (*D. borgmeieri*) over several generations in a laboratory is possible. This invites future research areas on the behavior and physiology of this species. It also means that mass-rearing of this species for managed pollination services may one day become possible. Secondly, we have updated our knowledge of the volatiles produced by flowers of this major crop.

By improving our understanding the chemical ecology of cacao pollination we are better placed to understand the poor pollination rates in many cacao plantations and relate this to midge-cacao interactions. Cacao pollination is understudied, with little known about the plant-insect interactions involved. Even less is known about pollination of wild trees (Chumacero de Schawe et al. 2018) in its native range (Amazon basin) (Richardson et al. 2015), although the importance of small Hymenoptera on wild cacao may have been underestimated in the past, compared to agroforestry systems where Diptera are usually anticipated to be most important (Entwistle 1972; Winder 1978a). The information we have acquired in this study can be further expanded and perhaps even used in future breeding programs.

There is increasing global interest developing in precision pollination systems, including via optimizing commercially reared pollinators (Molet et al. 2009), integrated pollination and pest management systems (Karise et al. 2016; Smagghe et al. 2013) and deployment of agricultural technologies in improvement of pollination (Olombria Ltd. 2019). This may open up future opportunities to use an improved understanding of non-bee pollinators in commercial settings to improve crop production, especially in high-value crops with growing global demand such as cacao.

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Figure legends

Fig 1 Relative amounts of compounds present in volatile collections from cacao flowers from GC-MS analyses on a non-polar DB5 GC column (the amounts of 17-triene were determined from analyses on a polar DBWax column; compound abbreviations: **5-13:H** 5-tridecene; **13:H** tridecane; **6+7-14:H** 6- and 7-tetradecene; **14:H** tetradecane; **Z7-15:H** (*Z*)-7-pentadecene; **E7-15:H** (*E*)-7-pentadecene; **15:H** pentadecane; **16 diene** diunsaturated 16-carbon hydrocarbon; **7+8-16:H** 7- and 8-hexadecene; **17 diene** diunsaturated 17-carbon hydrocarbon; **17 triene** triunsaturated 17-carbon hydrocarbon; **Z8-17:H** (*Z*)-8-heptadecene; **E8-17:H** (*E*)-8-heptadecene)

Fig 2 Cocoa midge preferences for (a) natural cacao flower odor and (b) synthetic cacao flower odor blend (SCFO) in a Y-tube olfactometer, with solvent control in the opposing arm. * indicates significant preference, $p < 0.05$.

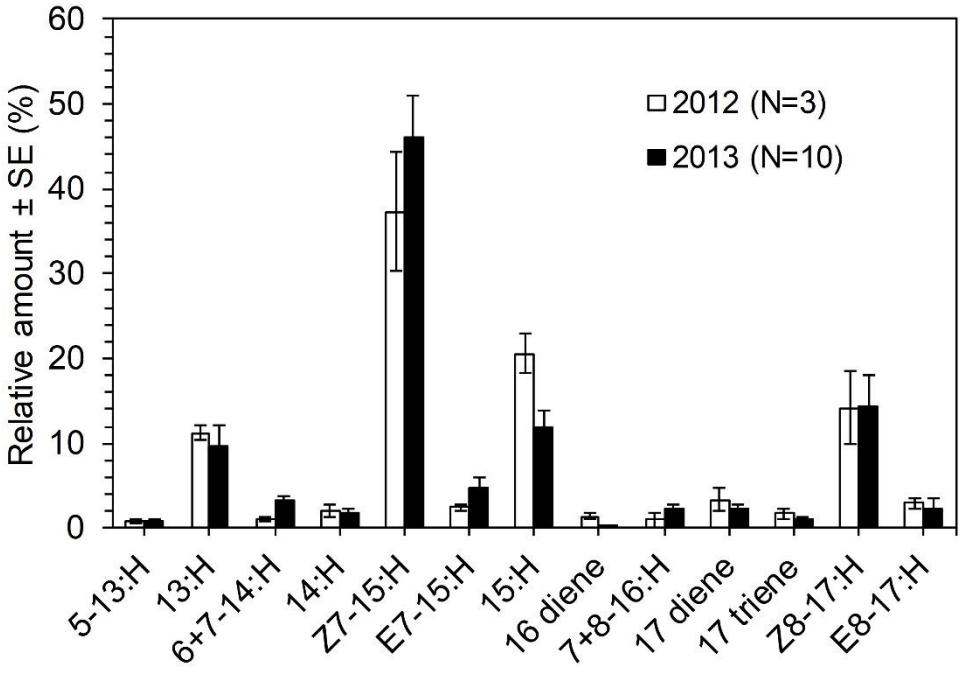

Fig 1 Relative amounts of compounds present in volatile collections from cacao flowers from GC-MS analyses on a non-polar DB5 GC column (the amounts of 17-triene were determined from analyses on a polar DBWax column; compound abbreviations: **5-13:H** 5-tridecene; **13:H** tridecane; **6+7-14:H** 6- and 7-tetradecene; **14:H** tetradecane; **Z7-15:H** (*Z*)-7-pentadecene; **E7-15:H** (*E*)-7-pentadecene; **15:H** pentadecane; **16 diene** diunsaturated 16-carbon hydrocarbon; **7+8-16:H** 7- and 8-hexadecene; **17 diene** diunsaturated 17-carbon hydrocarbon; **17 triene** triunsaturated 17-carbon hydrocarbon; **Z8-17:H** (*Z*)-8-heptadecene; **E8-17:H** (*E*)-8-heptadecene)

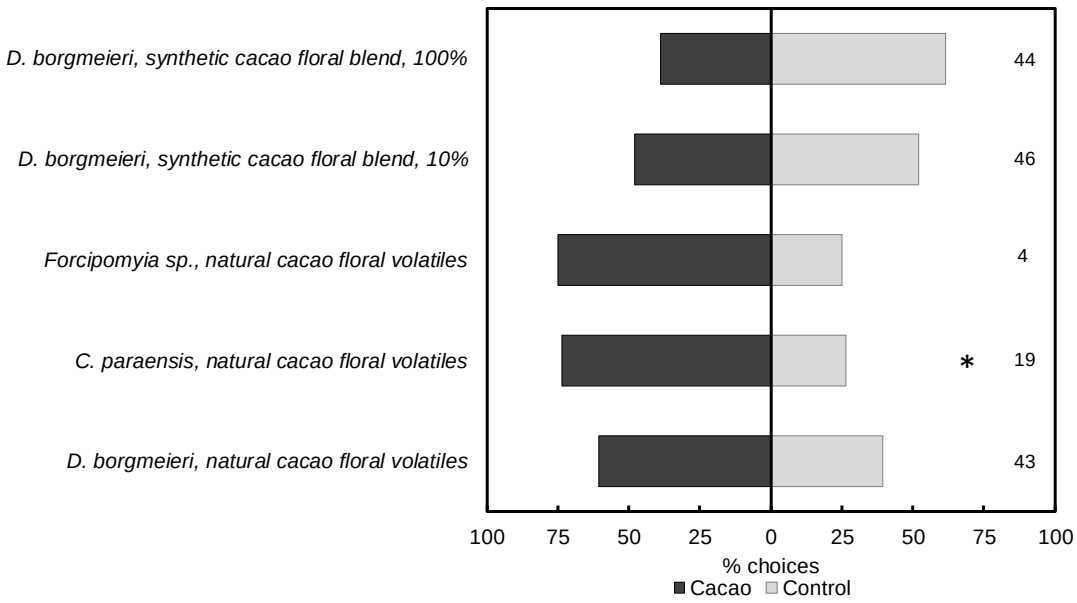

Fig 2 Cocoa midge preferences for the species tested, for natural cacao flower odor and synthetic cacao flower odor blend (SCFO) in a Y-tube olfactometer, with solvent control in the opposing arm. Figures on bars indicate sample size. * indicates $p < 0.05$ significant preference.

TABLE 1 Compounds identified in volatiles from cacao flowers and their GC retention indices (RI) on non-polar DB5 and polar DBWax columns, with retention indices and characteristic mass spectral ions of the dimethyldisulfide (DMDS) derivatives of unsaturated compounds

		Retention Index (RI)		DMDS Derivative	
	Compound[1]	DB5	DBWax	RI (VF5)	Ions (*m/z*)
1	5-13:H	1291	1321	1929	117, 159, 276
2	13:H	1300	1300		
3	6-14:H	1382	1426	2022	131, 159, 290
4	7-14:H	1384	1426	2019	145, 290
5	14:H	1400	1400		
6	Z7-15:H	1485	1521	2118	145, 159, 304
7	E7-15:H	1488	1521	2132	145, 159, 304
8	15:H	1500	1500		
9	16 diene	1576	1659	ND[2]	
10	7-16:H	1580	1619	2218	159, 318
11	8-16:H	1582	1619	2218	145, 173, 318
12	17 diene	1674	1758	2296	131, 199; 159, 171; 235, 282
13	17 triene	1679	1813	ND[2]	
14	Z8-17:H	1681	1719	2314	159, 173, 332
15	E8-17:H	1687	1719	2323	159, 173, 332

[1] Compounds numbered according to elution order on DB5 column; abbreviations: 13:H tridecane; Z5-13:H (*Z*)-5-tridecene; 17 diene diunsaturated 17-carbon hydrocarbon; 17 triene triunsaturated 17-carbon hydrocarbon; etc (see Fig. 1).

[2] ND not detected