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RESEARCH ARTICLE

More to legs than meets the eye: Presence and function of pheromone compounds on heliothine moth legs

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Abstract

Chemical communication is ubiquitous in nature and chemical signals convey species-specific messages. Despite their specificity, chemical signals may not be limited to only one function. Identifying alternative functions of chemical signals is key to understanding how chemical communication systems evolve. Here, we explored alternative functions of moth sex pheromone compounds. These chemicals are generally produced in, and emitted from, dedicated sex pheromone glands, but some have recently also been found on the insects' legs. We identified and quantified the chemicals in leg extracts of the three heliothine moth species *Chloridea* (*Heliothis*) *virescens*, *Chloridea* (*Heliothis*) *subflexa* and *Helicoverpa armigera*, compared their chemical profiles and explored the biological function of pheromone compounds on moth legs. Identical pheromone compounds were present on the legs in both sexes of all three species, with no striking interspecies or intersex differences. Surprisingly, we also found pheromone-related acetate esters in leg extracts of species that lack acetate esters in their female sex pheromone. When we assessed gene expression levels in the leg tissue, we found known and putative pheromone-biosynthesis genes expressed, which suggests that moth legs may be additional sites of pheromone production. To determine possible additional roles of the pheromone compounds on legs, we explored whether these may act as oviposition-deterring signals, which does not seem to be the case. However, when we tested whether these chemicals have antimicrobial properties, we found that two pheromone compounds (16:Ald and 16:OH) reduce bacterial growth. Such an additional function of previously identified pheromone compounds likely coincides with additional selection pressures and, thus, should be considered in scenarios on the evolution of these signals.

KEYWORDS
antimicrobial, chemical communication, heliothine moths, hexadecanal (16:Ald), multifunctionality, sex pheromone

1 | INTRODUCTION

1.1 | Chemical signals can have multiple functions

Chemical communication is the oldest and most abundant form of communication in nature (Wyatt, 2003). Insects especially use chemical signals to convey species-specific messages. Sex pheromones are examples of species-specific chemical signals and many nocturnal insects rely on sex pheromones to find conspecific mating partners (Allison & Cardé, 2016; Greenfield, 1981; Groot et al., 2016). Despite their specificity, chemical signals may have multiple functions. While sex pheromones can be used as mate recognition signals inhibiting cross-attraction between species, they may also function as defence mechanisms or provide information for other life stages. For example, in the Egyptian cotton leafworm *Spodoptera littoralis*, the female sex pheromone attracts males, but also lures larvae to food (Poivet et al., 2012). Similarly, the queen pheromone of the honey bee *Apis mellifera* not only attracts males, but also suppresses fertility in workers (Blum, 1996). Also, cuticular hydrocarbons (CHCs) form waxy protective layers on the cuticle in insects and prevent desiccation, but are species-recognition signals in social and solitary insects as well (Blomquist & Ginzel, 2021; Singer, 1998; for a recent review see Holze et al., 2021). Since signal evolution depends on the types of selection pressures, identifying the multiple functions of signals in different contexts is key to understanding how chemical communication systems evolve.

1.2 | Moth sexual signals may be shaped by multiple selection pressures

Moth sex pheromones have traditionally been recognized as species-recognition signals and thus to be involved in communication with other species. Signals that are involved in interspecies interactions are under natural selection. However, sex pheromones also convey information to individuals of the same species (Allison & Cardé, 2008; De Pasqual et al., 2021; Glover et al., 1991; Gonzalez-Karlsson et al., 2021; Johansson & Jones, 2007; Karlson & Butenandt, 1959; van Wijk et al., 2017; Zhu et al., 1997; Zweerus et al., 2021). These signals can thus also be under sexual selection. If a signal has multiple functions, several and possibly conflicting selection pressures shape its evolution.

1.3 | Sex pheromone compounds not only occur in moth pheromone glands but also on moth legs

Interestingly, moth sex pheromone compounds that are usually produced and present in the female sex pheromone gland were recently discovered on the legs of heliothine moths (Lepidoptera: Noctuidae) (Choi et al., 2016). Predominantly hexadecanal (16:Ald), a common minor gland component, was found on male and female tarsi of the corn earworm *Helicoverpa zea* (Boddie), the oriental tobacco budworm *Helicoverpa assulta* (Guenée), the cotton bollworm *Helicoverpa armigera* (Hübner) and the tobacco budworm *Chloridea* (formerly *Heliothis*) *virescens* (Fabricius) (Choi et al., 2016). In addition to 16:Ald, heliothine moth tarsi contain octadecanal (18:Ald) and various butyrate esters and hydrocarbons (Böröczky et al., 2008; Choi et al., 2016). Moths were found to deposit butyrate esters and hydrocarbons while walking (Choi et al., 2016).

1.4 | Are pheromone compounds on moth legs possibly produced in, or deposited on, leg tissue?

The origin of pheromone compounds on moth legs is unknown. Pheromone compounds could be locally produced in leg tissue or could be transferred due to grooming other parts of the body. For instance, American cockroaches *Periplaneta americana* remove cuticular hydrocarbons to enhance olfaction by grooming their antennae (Böröczky et al., 2013). Even though female moths evert the pheromone gland from the ovipositor to release pheromone, females are not grooming or moving their legs during this so-called 'calling' behaviour (NLZ pers. observation). Also, Choi et al. (2016) did not find 16:Ald in whole body extracts when legs were removed, which suggests that these chemicals are not present on the body surface due to grooming. Moreover, an enzyme responsible for 16:Ald biosynthesis is expressed in *H. zea* male tarsi (Dou et al., 2019, 2020). Therefore, the leg tissue could be a production site for pheromone compounds.

1.5 | Identifying the biological function of pheromone compounds on moth legs

The appearance of pheromone compounds together with other chemicals on heliothine moth legs suggests that these chemicals convey more messages than sex pheromone alone. Nevertheless, the biological function of the chemical compounds on the moth legs has remained unclear. Some Lepidopterans possess distinguished scent-organs on legs that emit pheromone (reviewed in Birch et al., 1990; Schulz et al., 1990; Weller, 1990). Males of the day-flying South American palm borer *Paysandisia archon* (Lepidoptera: Castniidae) possess glandular scent scales (androconia) on their legs to emit a sex pheromone (Frérot et al., 2013; Hamidi & Frérot, 2020; Jordan, 1923). In heliothine moths, however, neither males nor females have glandular structures on the legs. Choi et al. (2016) suggested that the chemicals on moth legs help in surface adhesion as aldehydes and also hydrocarbons facilitate surface adhesion in beetles (Attygalle et al., 2000; Geiselhardt et al., 2009) and locusts (Vötsch et al., 2002) but these suggestions have not been investigated further. The aim of this study was to explore additional functions of the chemicals found on moth legs.

In this study, we first analysed the pheromone compounds on legs of the three heliothine species *C. virescens*, *C. subflexa*, *H. armigera and* determined any intersex and interspecies differences in the pheromone profiles of legs. To determine whether moth legs

could be the production site of several pheromone compounds, we quantified the expression of candidate genes putatively involved in the moth sex pheromone biosynthetic pathway. We further tested the following three hypotheses on the biological function of pheromone compounds on the legs of heliothine moths:

1. Pheromone compounds on the legs play a role in moth sexual communication.

Analogous to pheromones from gland tissue, chemical signals from legs may play a role in sexual chemical communication. If chemical signals from legs are used for mate choice, we expected to find clear differences in amounts and/or composition of the pheromone compounds between male and female legs. Since moth sex pheromones are generally species-recognition signals, we also expected to find species-specific pheromone profiles in leg extracts.

2. The compounds from legs are used as host-marking pheromone

In addition to a function in sexual communication, the pheromone compounds on legs could act as marks on host plants and may signal the location of eggs to other females. This has been demonstrated in yucca moths, where such a host-marking pheromone from the female abdomen discourages other females from using the same oviposition site (Huth & Pellmyr, 1999). Since chemicals from legs are left on surfaces (Choi et al., 2016), females potentially leave chemical marks during oviposition. In heliothine moths, spatial spreading of the eggs is important for larval survival because the larvae of most species are cannibalistic (reviewed in Richardson et al., 2010). Therefore, we evaluated whether the chemicals from moth legs could function as oviposition-deterring signals.

3. Chemicals from moth legs have antimicrobial properties

Since chemicals from legs may be deposited at oviposition sites, we additionally hypothesised that they may protect eggs against microbial threats. Microbial infections reduce moth egg viability (Daquila et al., 2021; Hajek et al., 1996; Sikorowski et al., 2001; Tanabe & Tamashiro, 1967). Chemicals from moth legs could disinfect surfaces to prevent microbial growth. The external application of substances by the mother to protect eggs have been demonstrated in several insects. For instance, Japanese termites *Reticulitermes speratus* spread saliva containing antibacterial and antifungal compounds onto eggs (Matsuura et al., 2007; Matsuura & Matsunaga, 2015) and European earwigs *Forficula Auricularia* apply hydrocarbons by grooming the eggs to fight fungal growth (Boos et al., 2014). In Lepidoptera, females of the swallow tail butterfly *Byasa alcinous* coat their eggs with aristolochic acids to protect them from predators (Nishida & Fukami, 1989), and bella moths *Utetheisa ornatrix* protect the eggs by parental pyrrolizidine alkaloids (Dussourd et al., 1991; Eisner & Meinwald, 1995; Hare & Eisner, 1993). Therefore, we tested whether the main chemicals found on the moth legs can inhibit microbial growth.

2 | METHODS

2.1 | Insects

Chloridea virescens and *C. subflexa* populations were sourced from North Carolina State University, USA. and the Max Planck Institute for Chemical Ecology, Jena, Germany, while *H. armigera* was sourced from the School of Biological Sciences, The University of Queensland, Brisbane, Australia. All species were reared at the Institute for Biodiversity and Ecosystem and Dynamics (IBED), University of Amsterdam in climate chambers at 60% relative humidity and 25 ± 1°C with a photoperiod of 14 h light (photophase): 10 h dark (scotophase) photoperiod (lights off at 11 a.m. CET). We reared *C. virescens* larvae and *H. armigera* individually in plastic cups (37 mL, Solo, Lake Forest, Illinois), filled with pinto bean diet (Burton, 1970) and *C. subflexa* in identical cups but on a wheat germ/soy-flour-based diet (BioServ Inc., Newark, DE, USA). After pupation, we checked pupae daily for adult emergence, after which we sexed the adults and fed them with 10% sucrose solution provided through 1 cm cotton dental wick. The same environmental conditions were used for all experiments.

2.2 | Analysis in different species and two selection lines

To identify and quantify pheromone compounds on moth legs, we extracted chemicals from male and female legs of *C. virescens*, *C. subflexa* and *H. armigera*. In addition, we analysed the chemical profiles of males and females of two selection lines in *C. virescens*, which differ in an active or non-active delta-11-desaturase (*d11*) expressed in sex pheromone glands (Bjostad & Roelofs, 1983; Groot et al., 2019; Roelofs & Bjostad, 1984; Vogel et al., 2010). These selection lines were created based on the 16:Ald/Z11-16:Ald ratio in the female sex pheromone blend (for details see Groot et al., 2014, 2019), a so-called Low line and a High line: 'Low' females convert 16:Ald to the unsaturated counterpart (Z)-11-hexadecenal (Z11-16:Ald, the major sex pheromone component) and thus show a relatively low ratio of 16:Ald/Z11-16:Ald in their pheromone blend, which is the commonly described sex pheromone blend of *C. virescens*. Contrastingly, 'High' females have a stop codon in the first exon of delta-11-desaturase and thus show a high ratio of 16:Ald/Z11-16:Ald (Groot et al., 2014, 2019).

2.3 | Pheromone extraction from legs and GC analysis

To identify the chemical profiles of the legs of all three species and sexes, we extracted the chemical compounds from legs after at least 3 h into the scotophase (i.e., dark period), as follows. We removed all legs from an individual using watchmakers' forceps (Fine Science Tools, USA). We placed the six legs

of each individual in a glass insert filled with 50 μL hexane, in which 200 ng pentadecane (C15) was dissolved as internal standard for chemical analysis. To prevent cross-contamination, we cleaned the forceps in hexane between samples. After an incubation period of 30–60 min, we transferred the hexane solution to a new glass insert, using a 100 μL Exmire syringe (MSR100, ITO Company, Japan). We stored the extract in a 4 mL glass vial with a solid top polypropylene cap with a TFE seal at −20°C. We processed all samples within 2 weeks in a gas chromatograph (GC) for chemical analysis. The GC analysis was performed according to the protocol published in Groot et al. (2010) and summarized briefly here. The extracts were concentrated to 1–2 μL under a gentle flow of nitrogen. The concentrated samples were injected into a GC (Agilent Gas Chromatograph, HP7890) equipped with a high-resolution polar capillary column (DB-WAXetr) and a flame-ionization detector (FID) and analysed running the identical programme as described in Groot et al. (2010). To identify the chemical compounds on the legs, we used three synthetic pheromone blends that each contained all known sex pheromone compounds of the respective species. We made the synthetic multiple-component blends with pure compounds purchased from Pherobank (Pherobank BV, Wageningen). In addition, we used an alkane standard (C7–C40 alkane standard Sigma-Aldrich, 49 452-U) to identify long-chain compounds in the leg extracts. We integrated the GC signal using the ChemStation software (Agilent, Technologies Deutschland GmbH, Boblingen, Germany) and calculated the amount of each compound relative to the internal standard (C15). To determine whether there are significant differences in pheromone on legs between species and sexes, we conducted two-tailed *t*-tests with equal variances in a matrix format.

2.4 | GC–MS analysis to confirm the identities of the compounds

To confirm the identity of the peaks detected in the GC analysis, we ran leg extracts from male and female moths in GC–MS (Thermo Trace 1300 GC, Thermo Fisher Scientific) equipped with a mass-selective detector (Thermo Exactive Orbitrap MS) and a DB 5-MS column (30 m × 0.25 mm × 0.25 μm, Agilent J&W) and identified the compounds by comparing their retention times and mass spectra to those of authentic standards. The oven temperature was programmed at 50°C for 1.5 min, followed by an increase to 170°C (5°C/min), held for 2.0 min, followed by another increase to 320°C (5°C/min), held for 2.5 min. The injector temperature was kept at 250°C. We confirmed the identity of the peaks by checking for identical retention times of the peaks together with the above-mentioned standards and their mass spectra, and matching it with the National Institute of Standards and Technology (NIST) library search. In case of multiple library matches, we further checked for the masses of marker ions.

2.5 | Gene expression quantification (qPCR) of pheromone-biosynthesis genes in pheromone glands, hairpencils and front legs of *C. virescens*, *C. subflexa* and *H. armigera* males and females

To determine whether the pheromone compounds identified on the legs may also be produced in leg tissues, we quantified and compared the expression of three candidate genes putatively involved in acetate-ester production along with delta-11-desaturase (*d11*) in the following tissues: female sex pheromone glands, male hairpencils and female and male front legs. To obtain a comparison of the expression of the lesser-known candidate genes and the better-studied *d11*, we also determined the gene expression in the thorax tissue of one species (*C. subflexa*). The pheromone gland-specific *d11* is not expressed in *C. subflexa* thorax tissue and this tissue therefore acted as a negative control (Groot et al., 2016; Vogel et al., 2010).

To extract RNA from all the tissues, we collected front legs of both sexes, the pheromone gland of females, the hairpencils of males and the thorax from each individual in separate Eppendorf tubes. All samples were flash frozen in liquid nitrogen and stored at −80°C, after which we crushed the tissues with a cooled pestle. We extracted the RNA using the Jena Analytik innuPREP RNA mini kit 2.0 according to the manufacturer's protocol, including an 80% ethanol washing step. We determined the integrity of the RNA using 1.5% agarose gel electrophoresis and the quantified the RNA concentration using Nanodrop (ND1000, Thermo Fisher Scientific). We then diluted all RNA samples to a concentration of 10 ng/μL with Ultrapure MilliQ Water (Sigma-Aldrich).

To determine expression levels of sex pheromone biosynthesis-related genes, we conducted qPCR experiments with primers of the following genes: *d11*, carboxylesterase 21 (*cxe21*, also known as *HsTED*), a lipase (*lipX*) and another esterase (*est1*). We chose these genes because previous QTL analysis, RNA-Seq and CRISPR experiments have indicated that these genes play a role in the biosynthesis of sex pheromone compounds in heliothine moths (De Fouchier et al., 2022; Fruitet et al., 2022; Groot et al., 2009; Vogel et al., 2010). Since *cxe21*, *lipX* and *est1* have recently been identified as players in the synthesis and degradation of acetate esters (De Fouchier et al., 2022; Fruitet et al., 2022), we expected to find these genes expressed in tissues producing pheromone compounds. Primers for the target genes were designed using Primer 3 Web version (primer3_results.cgi release 4.1.0) (see Supporting Information). Primer efficiencies were evaluated based on dilution series. As reference gene, we used the housekeeping gene Eukaryotic translation Initiation Factor 4 alpha (*eIF4A*, Liu et al., 2016). The layout of the 96-well plates were such that we tested the four target genes (i.e., *d11*, *cxe21*, *lipX* and *est1*) plus the reference gene (i.e., *eIF4A*) in leg tissue and pheromone gland/hairpencil tissue of two males and two females of the same species on one plate (see Table S2). This allowed us to draw interspecies comparisons per plate based on two biological replicates per sex. Since we compared in total four species/lines and two

sexes, we ran a total of 10 plates (i.e., 3×*H. armigera*, 4×*C. virescens* High + Low, 3×*C. subflexa*). In addition, we ran two plates with thorax samples and female pheromone gland / male hairpencil tissue of *C. subflexa*. Gene expression levels were determined by calculating the number of target molecules expressed relative to 1000 of the reference gene.

To quantify the gene expression levels for each tissue, we pooled the RNA from all leg samples of each individual using equal amounts of each leg pair sample. We synthesized cDNA using the Verso cDNA Synthesis Kit with 10 ng/μL RNA (Thermo Fisher Scientific) according to the manufacturer's protocols and then tested for successful synthesis with PCR with Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific) on a 2% agarose gel. We then used 2 μL of the 4 ng/μL of the synthesized cDNA (Applied Biosystems 7500, Thermo Fisher Scientific) together with the HOT FIREPol EvaGreen qPCR Mix Plus Rox (Solis BioDyne) for qPCR analysis. We applied the following reaction times: activation at 95°C for 12 min, with 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C or 55°C for 20 s and elongation at 72°C for 20 s. All samples used in this report had a 70°C melt curve of one peak, and a no-template control with 0 fluorescence or 4 Ct values higher than the lowest Ct value of a sample on that plate. To assess gene expression levels in the different tissues, we calculated the mean expression levels (±SE) and plotted the data for visual comparison.

2.6 | Behavioural assay for testing pheromone compounds as oviposition-deterring signals

To test behavioural responses of ovipositing females to leg extracts, we made extracts as follows. We removed all six legs from adult moths that were 1–4 days old using watchmakers' forceps (Fine Science Tools, USA). We extracted the legs of 4–6 moths together in a glass insert filled with 50 μL hexane. After an incubation period of 30–60 min, we pooled the hexane solutions of up to 60 individuals in a new glass vial, using a 100 μL Exmire syringe (MSR100, ITO Company, Japan). To obtain a final concentration of 10 moth equivalents (ME) in 40 μL hexane, we calculated the required volume in relation to the number of moths the extract originated from and concentrated the liquid under a gentle flow of nitrogen.

To assess if compounds from legs have an oviposition-deterring effect, we tested how many females choose a control area compared to a site treated with leg extract to lay their first egg. The arena was constructed in insect cages (BugDorm 30 cm × 30 cm × 30 cm). The night prior to the assay, we mated the females to a non-sibling male. To test if compounds in leg extracts are oviposition-deterring signals, we applied the concentrated leg extract on filter papers (10 ME of leg extract in 40 μL hexane) using a 100 μL Hamilton syringe and let the filter papers dry in the fume hood. Additionally, we prepared two blank filter papers for two control areas and one hexane control (filter paper + 40 μL hexane) per arena.

For the assay, we visually divided the top of each cage into 4 equal cubic areas, numbered them and assigned a treatment to the area randomly. According to this pre-defined scheme, we placed the two control filter papers (blank), the hexane filter paper (40 μL hexane), and the filter paper with leg extract on top of the respective area. Testing was blind, meaning that the observer did not have any information during the experiment about the order of the treatments.

The observation started when the female was released in the bottom centre of a cage, which was 60 min before the onset of the scotophase. In each round, a total of 6 females were observed simultaneously. We observed the activity of each female and noted the time of event and the areas she oviposited eggs with a simple code (e.g., 'O3' for ovipositing in area 3). Since we aimed specifically to assess the first oviposition choice site, the observations lasted for up to 3 h (i.e., 2 h into the scotophase) or until a female oviposited more than 4 eggs in the cage. We froze all females after the experiment and checked them for a spermatophore to confirm the success of the initial mating. To evaluate the effect of leg extract on oviposition, we calculated the percentage of first-choices per treatment area and compared the values between the treatments using Fisher's exact test.

2.7 | Assessing antimicrobial properties of synthetic pheromone compounds

To assess antimicrobial properties of the pheromone compounds we found on moth legs, i.e. 16:Ald, hexadecenyl acetate (16:OAc), (*Z*)-11-hexadecenyl acetate (Z11-16:OAc) and hexadecanol (16:OH), we tested these compounds for their inhibitory effects on the growth of *Bacillus megaterium* (gram positive bacterium), *Escherichia coli* strain DH5α (gram negative bacterium) and the fungi *Aspergillus niger* and *Aspergillus nomiae*, which both naturally occur on the insect diet in the lab and were identified in collaboration with the Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands (MSc thesis L. de Jeu, 2020, unpubl.). To prepare solutions of synthetic pheromone at three concentrations, we diluted pure compound stocks (purchased from PHEROBANK (Wageningen, The Netherlands), Shin Etsu (Tokyo, Japan) and Bedoukian (Danbury, CT, USA)) in hexane (*n*-hexane, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to concentrations of 900, 300 and 100 ng/μL. We checked the solutions for purity and concentration by running a 1:10 dilution of the 100 ng/μL solution in the GC (Agilent Gas Chromatograph, HP7890) on a DB-WAXetr column (for method details see Groot et al., 2010).

We cultivated stocks of *B. megaterium* and *E. coli* strain DH5α on Lysogeny Broth Agar (LBA). To establish stocks of *A. niger* and *A. nomiae*, we isolated fungal spores from the larval diet and grew them on Malt Extract Agar (MEA) plates. To prevent bacterial growth in these stocks, we added the antibiotics penicillin and streptomycin (MEA-PS).

2.8 | Inhibition-zone assay

To test if 16:Ald, 16:OAc, Z11-16:OAc or 16:OH, can inhibit microbial growth, we tested synthetic pheromone compounds in inhibition-zone assays on Mueller-Hinton Agar (MHA) plates with 2% glucose medium (70 191, Sigma-Aldrich, Netherlands). Such inhibition-zone

assays are simple and effective measures of antimicrobial activity of a substance (e.g., Bauer et al., 1959; Heatley, 1944). In preparation for the assay with bacteria, we suspended one colony per bacterium (either *B. megaterium* or *E. coli* DH5α) in 5 mL LBA and incubated the solution at 37°C for 24 h at 170 rpm. The next day, we spread the bacteria equally onto MHA plates using a sterile cotton bud. In preparation for the assay with fungi, we collected fungal spores from stock plates and prepared spore suspensions. To obtain an equal suspension of spores in water, we added one drop (about 30 μL) of Tween20 (P9416, Sigma-Aldrich) to 5 mL demi water (Scott & Alderson, 1974) and poured the solution onto an agar plate. Subsequently, we pipetted the fungal solution from the plate into a sterile 15 mL tube and stored it at 5°C. To prepare the MHA plates with fungi for the experiment, we mixed the fungal solution well before spreading it equally onto MHA plates with a sterile cotton bud.

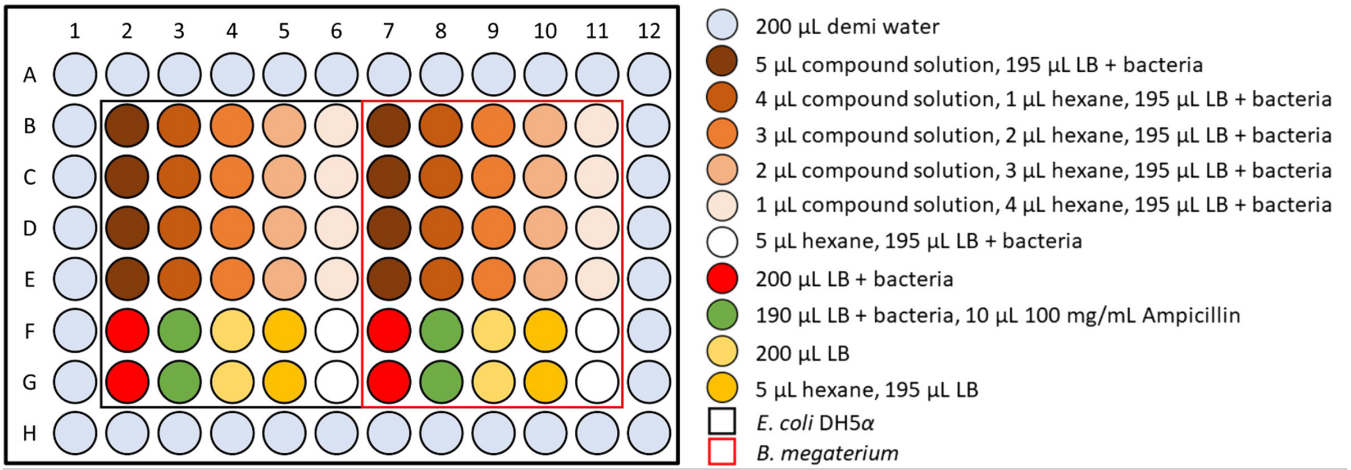
The inhibitory effect of the synthetic compounds was tested by applying 5 μL of each concentration (900, 300 and 100 ng/μL) of the compound solutions and a hexane control of 5 μL hexane directly onto each plate using a clean 10 μL syringe (Hamilton, UT). For all antibacterial assays, we applied 5 μL of Ampicillin (A9393, Sigma-Aldrich) directly to the plates as positive control. Due to different antibiotic sensitivities between the bacteria, a concentration of 0.05 mg/mL Ampicillin was used for *B. megaterium* and 5 mg/mL for *E. coli*. For all fungal assays, we used 5 μL 0.05 mg/mL Voriconazole (PZ0005, Sigma-Aldrich) directly applied to the plates as positive control. All plates were then incubated at 28°C, the bacterial plates for 24 h and the fungal plates for 48 h. We replicated the experiment 5 times for all four microorganisms. After incubation, we took pictures of every plate with a smart phone camera and determined the occurrence of inhibition zones as binary result (yes / no). We quantified the area of the inhibition zones in pixels/cm using the software ImageJ (version 1.53k, Schneider et al., 2012). To see if the treatments had a statistically significant effect, we analysed the data first by computing a one-sample *t*-test ($\mu = 0$). We then performed an ANOVA followed by a pairwise *t*-test assuming equal variances to determine if the inhibition was concentration-dependent.

2.9 | Bacterial growth assay

Since the inhibition-zone assay only assesses if a bacterium can grow under the given circumstances in a certain amount of time, but not how the bacteria grow, we also conducted a bacterial growth assay to determine the growth rate *r* and the maximum density (carrying capacity, *k*) of the bacterial culture in the given environment, which provides a quantitative measure of any inhibitory effects of the treatment. To quantify bacterial growth, we tested a range of concentrations of 16:Ald, 16:OAc, Z11-16:OAc and 16:OH in an optical density test on *B. megaterium* and *E. coli* DH5α in 96-well plates. To minimize effects of evaporation, we filled the outer wells with 200 μL demi water. We added either 5, 4, 3, 2 or 1 μL of the of the 900 ng/μL (in hexane) solution to the wells of one row, and topped up the wells by adding either 1, 2, 3, 4 or 5 μL of hexane (see example in Table 1), which resulted in the final concentrations of 0, 4.5, 9.0, 13.5, 18.0 and 22.5 ng/μL of each compound in the inner wells, in a final volume of 200 μL. The controls consisted of 200 μL of either LB only, 195 μL LB with 5 μL hexane, or LB with bacteria and 10 μL of 100 mg/mL Ampicillin (positive control). While we randomized the arrangement per row for each plate, all preparatory steps were identical for both *B. megaterium* and *E. coli*. Finally, we added the bacteria at OD600 = 0.05 (Schuurmans et al., 2009) to all the inner wells (Table 1). We sealed the 96-well plates with a breathable membrane (Breathe-Easy® sealing membrane, Sigma-Aldrich, Netherlands). To quantify bacterial growth, we measured the optical density in each well at OD600 over 14 h in a plate reader (SPECTROstar Nano®, BMG Labtech, Ortenberg, Germany). The machine read the plates every 10 min for 84 cycles at 37°C. Prior to each cycle, 300 seconds of double orbital shaking at 200 rpm was conducted. In total, each experiment was replicated 3 times (i.e., three 96-well plates per treatment were processed).

To compare growth rates among the different compounds and concentrations, we first calculated the net bacterial growth by subtracting the OD of the LB of every data point and analysed the data with the R package Growthcurver (Sprouffske & Wagner, 2016) in R and R studio (version 4.0.5, R Core Team, 2021). We modelled the

TABLE 1 Plate layout for the bacterial growth assay. In this illustration, the synthetic compounds are added to row B–E of the 96-well plate. The different colours represent different treatments at different concentrations.



population growth logistic equation based on the following formula (Sprouffske & Wagner, 2016):

$$N_t = k / \left(1 + \left(\left(k - N_0\right) / N_0\right) * \exp(-r * t)\right)$$

where N_t is the population size over time (t), k is the maximum population size (carrying capacity), and r is the population growth rate. We determined the carrying capacity k and growth rate r in each replicate before computing the average of k and r for every compound and concentration. To determine a differential effect of the pheromone compound or concentration on the bacterial growth rate and carrying capacity, we compared the average growth rate and the average carrying capacity in every treatment to the growth values of the control, using the non-parametric Kruskal–Wallis test with a Dunn's test as post-hoc, as assumptions for a parametric test were not met.

3 | RESULTS

3.1 | Pheromone compounds in heliothine moth leg extracts

We identified four pheromone compounds in the leg extracts, namely 16:OAc, Z11-16:OAc and 16:OH (Figure 1a; Supporting Information Figures S1) in addition to the previously reported compound 16:Ald (Choi et al., 2016). All these compounds were present in both sexes of all three species. While the relative amounts of each compound were similar in males and females for *C. subflexa* and in the *C. virescens* Low and High selection line, in *H. armigera* the relative amount of 16:Ald was higher in males than females ($t(26) = 3.18$, $p = 0.004$), while the relative amounts of 16:OAc ($t(26) = -2.71$, $p = 0.012$) and Z11-16:OAc ($t(26) = -3.07$, $p = 0.005$) were higher in females than males (Figure 1a). The total amounts of pheromone compounds on legs also differed between the sexes in *H. armigera*, where females had on average a significantly lower total amount (118.4 ± 21.0 ng) than males (368.2 ± 54.9 ng) ($t(26) = 4.25$, $p \leq 0.001$) (Figure 1b). The Low *C. virescens* selection line also contained significantly lower levels in the total amounts than the High *C. virescens* selection line (Figure 1b).

In addition to the known pheromone compounds, two long-chain carbon compounds, C25 and C27, were present in all samples. While *C. virescens* High line females had significantly higher amounts of C25 compared to males ($t(22) = 4.37$, $p \leq 0.001$), female leg extracts of all species consistently contained higher amounts of C27 than males (Figure 1c).

3.2 | All target genes are expressed in several tissues, including legs, of both sexes in multiple species

In line with previous findings that *d11* is specifically expressed in moth sex pheromone glands (Vogel et al., 2010), *d11* was mostly expressed

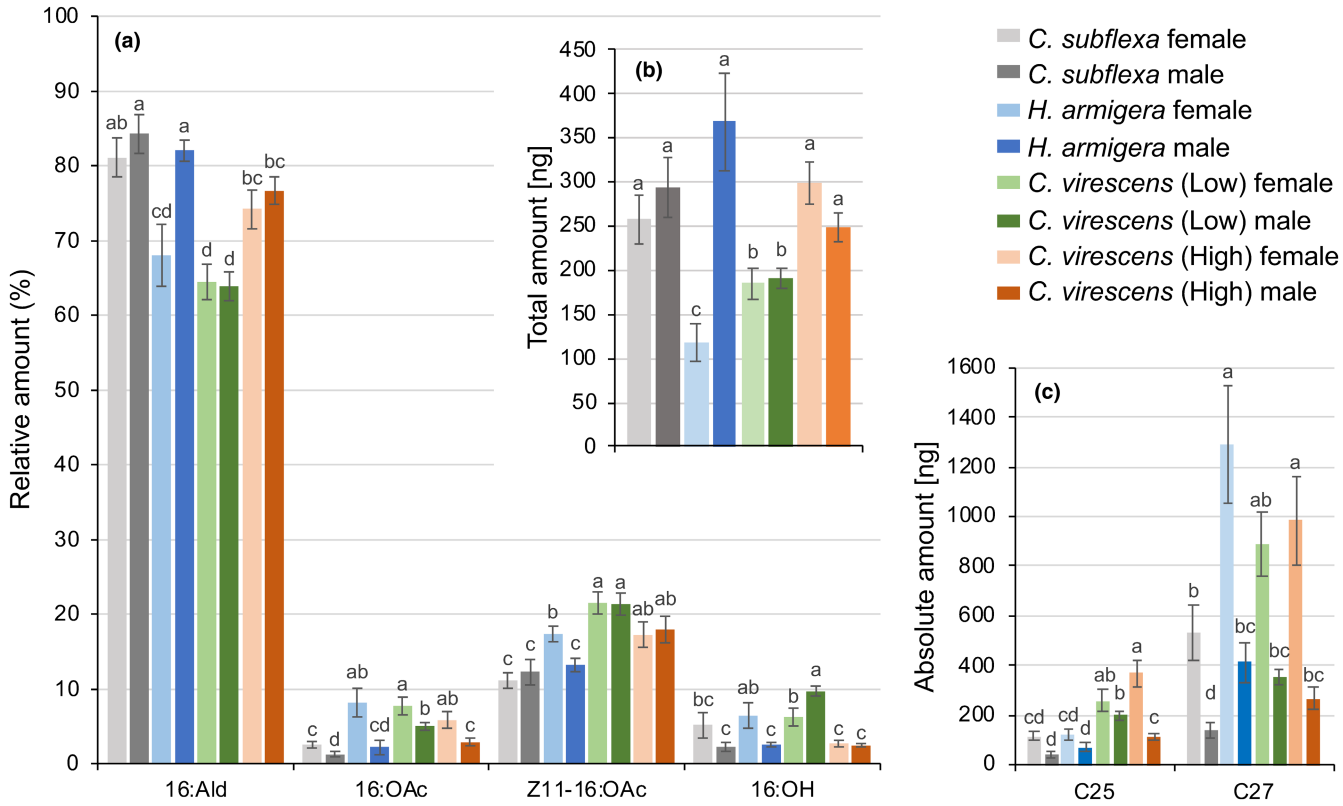


FIGURE 1 Average amounts (±SE) of pheromone compounds on heliothine moth legs. Bars show (a) average relative. amounts and (b) the total amounts of the four pheromone compounds and (c) the absolute amounts of long-chain carbon compounds. Different letters above the bars indicate significant differences.

in the female pheromone gland in all species (Figure 2a–e) but not in the thorax tissue that we used as negative control (Figure 2e). In addition, we found *d11* to be expressed in the male hairpencils of all species, which are known to produce biosynthetically related sex pheromone compounds (Hillier & Vickers, 2004; Teal & Tumlinson, 1989) (Figure 2a–d). Even though the thorax tissue worked as negative control for *d11* (Figure 2e), we found *cxe21* and *est21* to be expressed in thorax tissue of *C. subflexa* (Figure 2j,o).

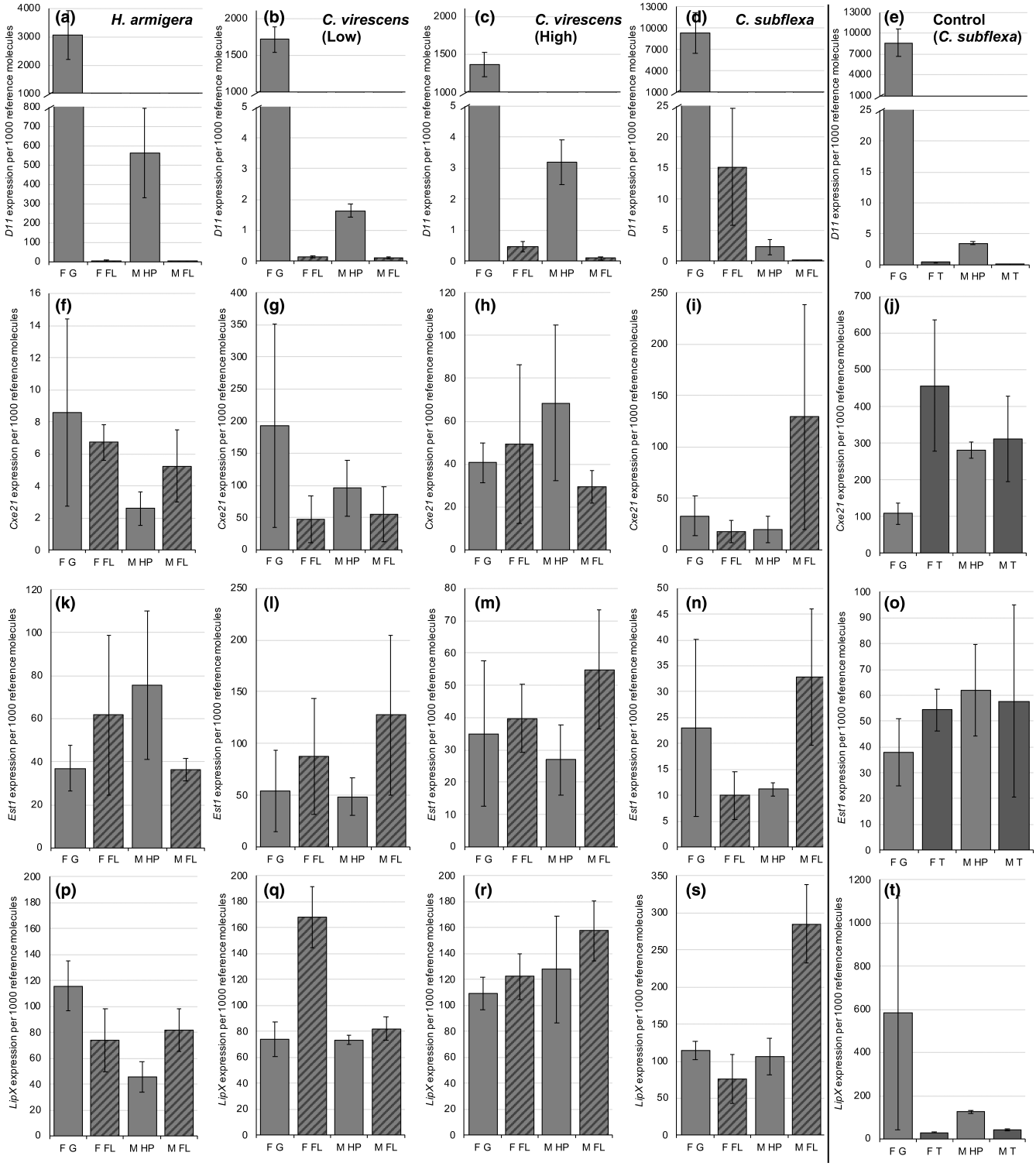


FIGURE 2 Mean gene expression levels (±SE) in several tissues and both sexes. FG, female gland; F FL, female front leg; M HP, male hairpencils; M FL, male front leg. Shaded bars = leg samples. Each column displays the data of one species, including *C. virescens* (Low) = *C. virescens* Low line, *C. virescens* (High) = *C. virescens* High line. The vertical line to the right separates the control column showing the gene expression relative to the control tissue (thorax), which was our negative control for d11. F T, female thorax; M T, male thorax.

Interestingly, *d11* was hardly expressed in the legs any of the species, with the exception of the leg tissue of *C. subflexa* females (Figure 2d). For the other genes, we found *cxe21*, *est1* and *lipX* to be expressed in leg tissue, with sometimes strikingly higher expression in male leg tissue compared to the other tissues. For instance, we found relatively high expressions of *cxe21* in male leg tissue of *C. subflexa* (Figure 2i). Moreover, *est1* showed the highest expression in male leg tissue of *C. virescens* Low (Figure 2l) and High (Figure 2m) and *C. subflexa* (Figure 2n). *LipX* was mostly expressed in female front legs of *C. virescens* Low (Figure 2q) and *C. subflexa* male leg tissue (Figure 2s).

3.3 | Chemicals from legs are unlikely oviposition-deterring signals

In testing whether leg extracts are an oviposition-deterring signal, we found that 12 out of 21 females laid their first egg into a control area with a plain filter paper (blank 1 and 2), which means that 28.6% of first eggs were laid in one of the two untreated areas. Five females (23.8%) oviposited their first egg in the hexane control area, and 4 females (19.1%) laid the first egg in the area treated with leg extract (Figure 3). Since these results did not show any significant differences in female oviposition-site choice (Fisher's exact test, $p = 0.94$), we conclude that leg extracts have no oviposition-deterring effect.

3.4 | Synthetic pheromone compounds show antimicrobial properties

3.4.1 | Inhibition-zone assay

In our test to assess antimicrobial properties of leg compounds, we found that fungal growth of *A. niger and A. nomiae* was unaffected by 16:Ald, 16:OAc, Z11-16:OAc and 16:OH (Supporting Information Figures S5 and S6, Table S2). However, the bacterial growth of *B. megaterium* (gram positive) and *E. coli* (gram negative) was inhibited by 16:Ald (Figure 4, Figures S7 and S8, Table S2) as all treatments showed a significant inhibition zone (all $p < 0.05$). Specifically, the lowest concentration of 100 ng/μL 16:Ald already inhibited the growth of both bacterial species (pairwise *t*-test, all $p < 0.001$), with even significantly stronger effects at 300 ng/μL (all $p < 0.001$) and 900 ng/μL (all $p < 0.001$) (Figures 4, S7 and S8). The hexane control and other pheromone compounds (i.e., 16:OH, 16:OAc and Z11-16:OAc) showed no inhibitory effect (Supporting Information Figures S5–S8, Table S2).

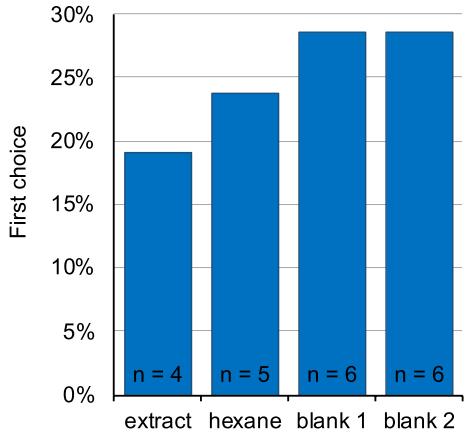


FIGURE 3 First choice for oviposition site.

3.4.2 | Bacterial growth assay

The quantification of the growth rate (*r*), which is the speed at which bacterial cells replicate, and the carrying capacity (*k*) of *B. megaterium* and *E. coli* showed that 16:Ald and 16:OH significantly inhibited the growth of both bacteria (Figure 5).

3.4.3 | 16:Ald and 16:OH reduced the growth rate of bacteria

The growth rate *r* of *B. megaterium* was significantly negatively affected by 16:Ald in a concentration-dependent manner. Increasing concentrations of 16:Ald had a stronger inhibitory effect (Figure 5a) and significantly reduced growth rates at concentrations of 18 ng/μL (Dunn's-test, $p = 0.042$) and 22 ng/μL ($p = 0.022$) compared to the control (Figures 5a and S9, Table S3). While the growth rate *r* of *E. coli* was not significantly reduced by 16:Ald, 16:OH significantly reduced *E. coli* growth at a concentration of 22.5 ng/μL ($p = 0.045$) (Figure 5b). The growth rate of both bacterial species was unaffected by the acetate esters (i.e., 16:OAc and Z11-16:OAc) (all $p \geq 0.05$) (Figures 5, S9, and S10, Table S3).

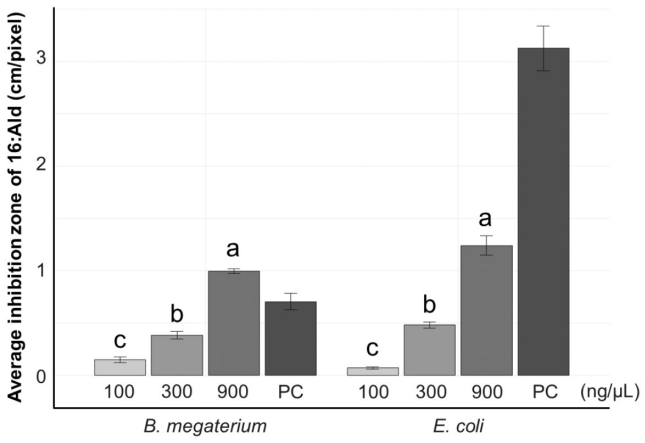


FIGURE 4 Inhibition of *B. megaterium* and *E. coli* by 16:Ald at three concentrations and the positive control (PC) with ampicillin. All inhibition zones were significantly different from zero. Different letters above the bars indicate significant differences in inhibition between different concentrations of 16:Ald, compared within one bacterium. Error bars = ±SE.

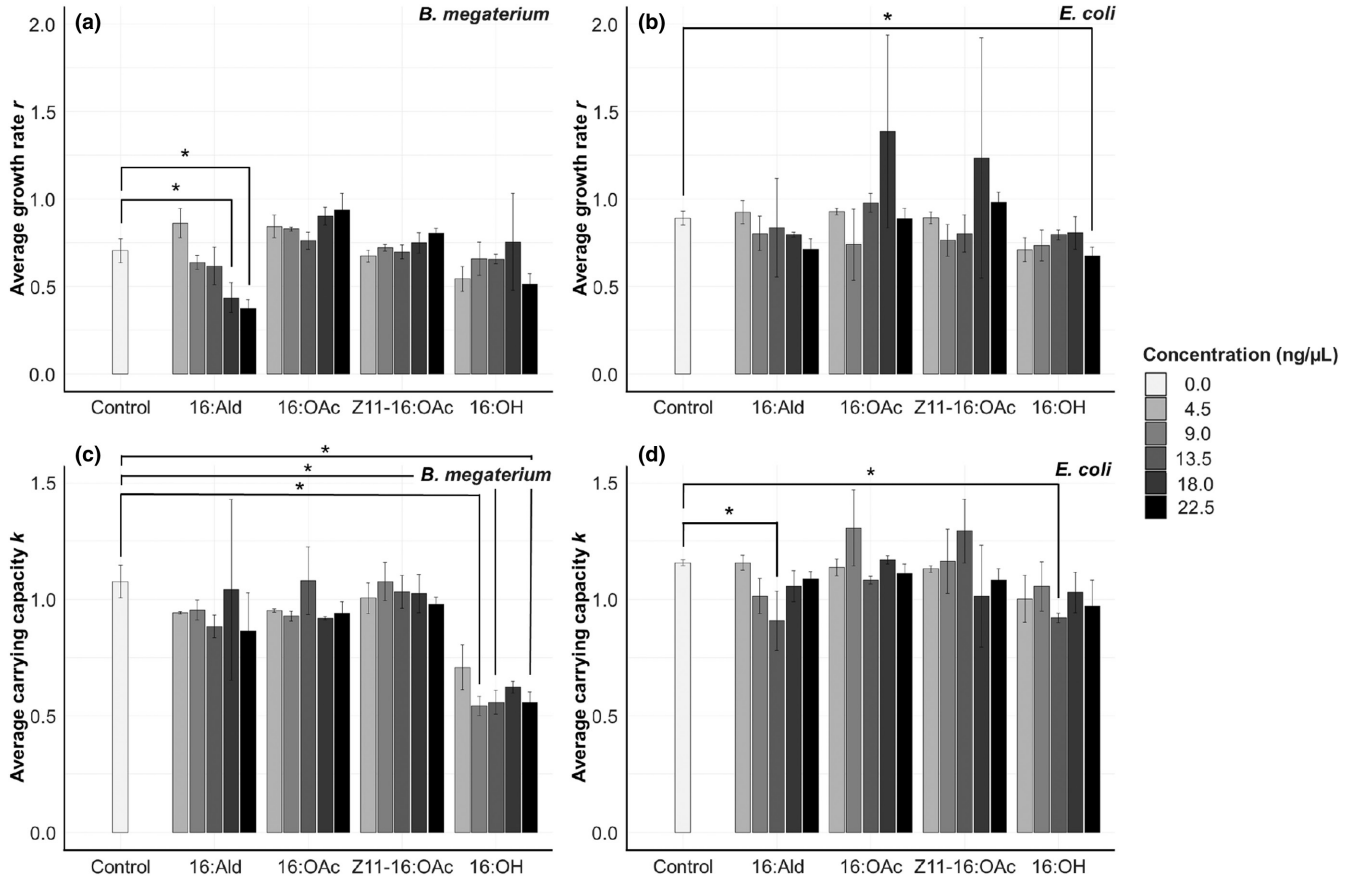


FIGURE 5 Mean growth rates (*r*) and carrying capacities (*k*) (±SE) of *B. megaterium* (a, c) and *E. coli* (b, d) exposed to different concentrations of four synthetic pheromone compounds. Increasing grey colours indicate increasing compound. concentrations. $^*p \leq 0.05$.

3.4.4 | 16:OH lowered the carrying capacity of bacteria

In addition to changes in growth rate *r*, 16:OH significantly decreased the carrying capacity *k* of *B. megaterium* at 9.0 ng/µL ($p = 0.016$), 13.5 ng/µl ($p = 0.020$) and 22 ng/µL ($p = 0.023$) (Figures 5c and S9, Table S3). The carrying capacity *k* of *E. coli* was significantly reduced by 16:Ald at a concentration of 13.5 ng/µL ($p = 0.040$) (Figure 5c) and by 16:OH at a concentration of 13.5 ng/µL ($p = 0.014$) (Figures 5d and S10, Table S3). Counterintuitively, higher concentrations of 16:OH (i.e., 18 and 22.5 ng/µL) did not affect *k* of *E. coli* ($p \geq 0.05$). The acetate esters (i.e., 16:OAc and Z11-16:OAc) did not have inhibitory effects on the carrying capacity of *B. megaterium* (all $p \geq 0.05$) (Figure 5c, Table S3) or *E. coli* (all $p \geq 0.05$) (Figure 5d, Table S3).

4 | DISCUSSION

Despite their specificity, chemical signals may not be limited to only one function. In exploring alternative functions of moth sex pheromone compounds that are present also on the legs, we analysed leg extracts of *C. virescens*, *C. subflexa* and *H. armigera*, and found 16:Ald, Z11-16:OAc and small amounts of 16:OAc and 16:OH without any sex- or species-specific patterns. We did find putative pheromone-biosynthesis genes expressed in the leg tissues, which suggests that moth leg tissue may produce these pheromone compounds. Our most interesting finding is that 16:Ald and 16:OH have antibacterial properties. Below, we discuss our results in more detail.

4.1 | Additional compounds but in lower amounts are present on moth legs than previously reported

Corresponding to the finding of Choi et al. (2016), we found 16:Ald to be the most abundant compound in the leg extracts of all three species. However, while Choi et al. (2016) reported 16:Ald in the range of milligrams on male tarsi of *Helicoverpa zea* and *H. armigera*, we detected much lower amounts in *Chloridea virescens*, *C. subflexa* and *H. armigera* in our extracts, with maximally 594 ng. It is possible that *Helicoverpa* species produce more 16:Ald on legs than *Chloridea* species (formerly *Heliothis*). The function of such high amounts of 16:Ald on legs remained unclear due to a lack of behavioural assays. Choi et al. (2016) proposed it to be a sexual signal, yet the behavioural relevance of 16:Ald is debatable and it may not be a sex pheromone component at all (Groot et al., 2005, 2010; Teal et al., 1986; Vetter & Baker, 1983).

In addition to 16:Ald, we consistently found 16:OH and two acetate esters (16:OAc and Z11-16:OAc) in all leg extracts. The presence

of 16:OH in leg extracts could be explained by the fact that this is a precursor of 16:Ald; alcohols applied to pheromone glands get converted into aldehydes (Fang et al., 1995; Luxová & Svatoš, 2006; Teal & Tumlinson, 1986, 1987, 1988). Even though 16:OH may be present in leg tissue, this primary alcohol is likely rapidly converted to 16:Ald (Teal & Tumlinson, 1986). We might thus have detected 16:OH in leg extracts while it is not emitted from legs. Since we did not find any interspecies or intersex differences in sex pheromone compounds on legs, we consider it unlikely that these compounds have a function in species recognition or intraspecific mate choice in the three heliothine species studied here. Whether chemical compounds on moth legs nevertheless play a role in partner search or attraction in a non-species- or sex-specific way remains an open question.

4.2 | Unexpected acetates are produced but likely also converted or degraded in and on legs

Since the female sex pheromone of *C. subflexa* contains unsaturated acetate esters, including Z11-16:OAc, whereas acetate esters are absent from the female sex pheromone of *C. virescens* and *H. armigera* and repel the males of both species (Gao et al., 2020; Groot et al., 2006; Vickers & Baker, 1997; Xu et al., 2016), we were surprised to discover acetate esters in leg extracts of both sexes and all species. Acetate esters have also been found in the hairpencils of *C. virescens* (Teal & Tumlinson, 1989) and the absence of acetate esters in the female sex pheromone obviously does not mean that the species is not able to produce these compounds, which was already suggested by Teal and Tumlinson (1987). This may explain our finding that three genes putatively involved in acetate biosynthesis were expressed in leg tissue of both sexes and all species. Since acetate degrading enzymes are present on legs of heliothine moths (Ding & Prestwich, 1986), acetate esters that are synthesized in legs could immediately be converted before being released into the environment.

4.3 | C25 and C27 show intersex differences and might be used for sexual communication

In contrast to the similar chemical profiles of the four known sex pheromone compounds, we found more C25 on female legs than male legs in the *C. virescens* High line and females of all species and lines investigated contained more C27 on legs than males. Less volatile compounds of 21 or more carbons usually function as short-range or contact pheromones (Jurenka, 2004). Contact sex pheromones are used in various insects, such as fruit flies, tsetse flies, beetles, cockroaches, termites, ants and bees (reviewed in Hanks & Millar, 2016; Howard & Blomquist, 2005; Ingleby, 2015; Schal et al., 1984; Singer, 1998). Cuticular lipids with a chain length of 25 and 27 carbons were previously detected on the forelegs of *C. virescens* males and females, with much higher amounts on female than on male forelegs (Böröczky et al., 2008). Choi et al. (2016) also extracted C25 and C27 butyrate esters and hydrocarbons from tarsi of *H. zea*, but with higher amount of butyrate esters on male than on female tarsi. Whether the reported long-chain compounds are identical across all three studies needs to be confirmed. The repeatedly detected intersex differences in long-chain carbon compounds on the legs suggest that hydrocarbons could play a role in sexual chemical communication of heliothine moths.

4.4 | Pheromone compounds found on legs could be produced in leg tissue

Since we found several genes expressed in the moth legs that are (putatively) involved in pheromone biosynthesis, moth leg tissue could be a pheromone production site. Although the thorax tissue serves as negative control for *d11*, we found the other genes also expressed in thoracic tissue of *C. subflexa*. Moreover, the quantitative expression between the *C. subflexa* samples differed (see Figure 2). This may be caused by the batched qPCR experiment due to the plate design and large individual variation. It is therefore impossible to derive any quantitative statements about the expression of the genes.

The genes *cxe21*, *est1* and *lipX* have recently been identified as putative genes involved in sex pheromone metabolism in *C. subflexa* (De Fouchier et al., 2022; Fruitet et al., 2022). As these candidate genes may be involved in acetate-ester synthesis as well as acetate-ester degradation (De Fouchier et al., 2022; Fruitet et al., 2022), we refrain from drawing any conclusions about their role in pheromone biosynthesis and considered our analysis as a first indication that pheromone-biosynthesis genes are also expressed in moth leg tissue. Dou et al. (2019, 2020) already demonstrated that 16:Ald is locally produced in legs of *H. zea*. Likewise, several pheromone-biosynthesis genes are expressed in leg tissue of the diamondback moth *Plutella xylostella* (He et al., 2017). Interestingly, we found delta-11-desaturase (*d11*) to be expressed in *C. subflexa* female legs, even though this enzyme was previously thought to be specific for female moth sex pheromone glands (Bjostad & Roelofs, 1983; Roelofs & Bjostad, 1984; Vogel et al., 2010). The expression of an acetate esterase and acyltransferase in addition to *d11* corroborates the notion that moth leg tissue may be production sites of pheromone compounds.

4.5 | Pheromone compounds are unlikely oviposition-deterring signals

Since moth legs come into contact with leaf surfaces during oviposition, we hypothesised that females could use the leg compounds to mark their oviposition sites. Such markings could signal the presence of eggs to other females and thus may lower the risk of cannibalism, which is prevalent in these species (Richardson et al., 2010). However, we did not find any deterrent effect of leg extracts on female oviposition-site choice in our experiments. We acknowledge

two potential constraints of the oviposition assay. First, due to the labour-intensive observations and the large number of individuals needed for the test of one extract, only a small sample size could be realized. Second, we used natural extracts at only one concentration for our assays, whereas the use of synthetic blends or higher concentration might reveal an oviposition-deterrent effect. Future experiments could explore a larger variation in treatments while sticking to biologically meaningful conditions. Another possibility is that chemicals from other body parts than the legs may have an oviposition-deterrent function.

4.6 | Pheromone compounds from moth legs could be used for chemical espionage

The chemicals released from legs could also provide information to other species. For example, chemical signals released by moths before or during oviposition can be exploited by egg parasitoids. The parasitoid wasp *Trichogramma pretiosum* attacks the eggs of *C. virescens* and other heliothine moths (Andrade et al., 2011; Lewis et al., 1976). In a closely related species, *T. chilonis*, females have been found to perceive 16:Ald and to show a positive dose response to multiple moth sex pheromone compounds (Boo & Yang, 2000). Leaf surfaces likely contain traces of chemicals left behind from moth legs (Choi et al., 2016) because moth sex pheromone can be adsorbed to plant surfaces (Fatouros et al., 2008; Noldus et al., 1991). Chemicals shed from moth legs could thus be used for chemical espionage. If this is the case, lower amounts of the leg compounds would be advantageous for moths.

4.7 | Pheromone compounds are antibacterial agents

Our most interesting results are the inhibitory effects of 16:Ald and 16:OH on the growth of both gram positive and gram negative bacteria as our results indicate that 16:Ald and 16:OH are natural antibiotics. Antimicrobial properties of several other alcohols and aldehydes have already been identified (reviewed in McDonnell & Russell, 1999; Mehranian et al., 2017). For example, glutaraldehyde is a popular and potent disinfectant and shows strong antibacterial properties (e.g., towards *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis*). In termites, eggs emit an egg-recognition pheromone that informs workers to take care of them, while the same substance protects the eggs from invading microbes (Matsuura, 2012; Matsuura et al., 2007). To our knowledge, the current study provides the first evidence that sex pheromone compounds of heliothine moths have an antibacterial function.

4.8 | The evolution of pheromone compounds is shaped by sexual and natural selection

In summary, we found that cuticular hydrocarbons found on the legs of three moth species may be used for sexual communication, while the previously identified sex pheromone compounds on the legs do not show any particular sex- or species-specific patters and are therefore unlikely involved in mate choice. Since we demonstrated that some of these sex pheromone compounds prevent bacterial growth, these compounds are most likely subject to both sexual and natural selection. Together, our study sheds light on the biological function of pheromone compounds on moth legs and shows that some of these compounds are relevant in different situations. Since these alternative functions suggest that additional selection pressures are present, our findings should be taken into account in developing scenarios on the evolution of these chemical signals.

AUTHOR CONTRIBUTIONS

Naomi L. Zweerus: Conceptualization (equal); formal analysis (equal); investigation (lead); methodology (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Laura J. Caton:** Formal analysis (supporting); investigation (equal); visualization (supporting); writing – review and editing (equal). **Lotte de Jeu:** Formal analysis (supporting); investigation (equal); visualization (supporting); writing – review and editing (supporting). **Astrid T. Groot:** Conceptualization (equal); funding acquisition (lead); investigation (equal); project administration (lead); resources (lead); supervision (lead); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

None declared.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jeb.14173.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in DRYAD at https://doi.org/10.5061/dryad.kh18932bd.

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