Semiochemicals mediating host-searching behavior of the ectoparasitoid *Holepyris sylvanidis* and their relevance for integrated pest management of stored-product insects

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Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently

and that I have not used any sources and aids other than those indicated by me.

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SAS, MH, and BF conceived the study and planned the experiments. SAS performed the experiments and analyzed the data. SAS wrote the first draft of the manuscript. BF and MH revised it, and all authors agreed with the final version.

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Summary

Summary

This thesis focused on the semiochemicals mediating host-searching behavior of the ectoparasitoid *Holepyris sylvanidis* (Hymenoptera: Bethylidae) which attacks larvae of various stored-product pest beetles, especially those of the confused flour beetle, *Tribolium confusum* (Coleoptera: Tenebrionidae). Prior to this thesis, it was already known that the parasitoid uses host-specific compounds released from larval feces of *T. confusum* for host location and that the attractiveness of host-specific kairomones for the parasitoid increases by the addition of habitat background odor. Moreover, host cuticular hydrocarbons (CHCs) serve as contact kairomones for host recognition by *H. sylvanidis* and mediate trail-following behavior in foraging parasitoids, but only for a period of two days. Despite these initial findings, many chemical and ecological aspects of the parasitoid's host search and the potential of host-specific kairomones to enhance biological control were still poorly understood.

Therefore, the goal of this thesis was to gain deeper insights in the chemically mediating host search of *H. sylvanidis*. Various behavioral bioassays and chemical analysis of host kairomones via coupled gas chromatography-mass spectrometry were performed to answer the following questions:

(i) Which factors drive the loss of kairomonal activity of host larval CHC trails over time? (Chapter 2)

(ii) Do *H. sylvanidis* females discriminate a host from a non-host species based on a host-specific CHC pattern present on the cuticle? (Chapter 3)

(iii) Does the additional application of host-specific kairomones improve the host search activity of the parasitoid and thus, its efficiency in controlling *T. confusum*? (Chapter 4).

In Chapter 2, we first investigated whether the time-limited kairomonal activity of host trails was due to changes in the CHC composition induced by microbes. Chemical analysis revealed that the CHC profile of host trails did not change qualitatively or quantitatively over a two-day period regardless of whether they were analyzed under sterile or non-sterile conditions. These results prompted the suggestion that factors other than microbial degradation might determine the temporary behavioral activity of CHC trails. This assumption was confirmed by our cryo-scanning electron microcopy results. Within two days, host trails gradually formed solid microstructures most likely due to a self-assembly of CHCs. But this process was reversible by hexane application which in turn led to the recovery of kairomonal activity of two-day-old CHC trails. Parasitoids followed these re-activated host trails as they did when encountering freshly laid ones. Our results suggest

that the gradual solidification of long-chain CHCs in a host trail over time reduces the perceptibility for *H. sylvanidis* as such that the trail is no longer followed.

In Chapter 3 we examined the influence of different CHC profiles of three closely related and one distantly related beetle species on the host recognition behavior of *H. sylvanidis.* In bioassays the parasitoid successfully recognized and accepted larvae of *Tribolium* spp. as hosts, whereas larvae of *Oryzaephilus surinamensis* were rejected. The latter species elicited a behavioral response in *H. sylvanidis* only when solvent extracted larvae were treated with a sample of *T. confusum* larval CHCs. Chemical analysis of larval extracts showed that the CHC profiles of the host and non-host species differed in their compositions. The main difference was the presence of methylated alkanes (MeAL) on the cuticle of *Tribolium* spp. and the absence of MeAL on that of *O. surinamensis*. MeAL serve as host recognition cues that enable the parasitoid to distinguish a host from a non-host species.

In Chapter 4, we first examined whether volatiles associated either to *T. confusum* larvae or to other host stages (e.g., beetle adults) participate in the host search of *H. sylvanidis* over long distances. The parasitoid was most attracted to a mix of two larval key compounds (2CM = (*E*)-2-nonenal and 1-pentadecene) emitted from larval feces, but the behavioral response of the parasitoid was concentration dependent. In contrast, volatiles released by *T. confusum* adults were not attractive for the parasitoid in all concentrations tested. We then studied whether the additional presence of synthetic 2CM (in the most attractive concentration) has an enhancing effect on the host search of *H. sylvanidis.* In three-day lasting flight cage experiment we offered *T. confusum* larvae either with additionally applied 2CM (test) or without (control) to parasitoid females. In test cages, parasitoids removed significantly more beetle larvae from the initial population within 48 h and parasitized a significantly higher number of host larvae after 72 h compared to those in the control cages. Furthermore, significantly more parasitoid offspring emerged from host larvae parasitized in the presence of 2CM. However, the increasing population size in the F1 was due to a significant increase in male offspring. These results illustrate that the host-finding success and parasitization rate of parasitoids can be improved by the additional release of host-specific, synthetic kairomones. But this is associated with changes in the population composition (male/female) of the parasitoid progeny.

Overall, the results obtained in this thesis on the semiochemicals mediating host-searching behavior of a natural antagonist of stored-product pest beetles contribute to a better understanding of the interaction between parasitoids and their host species. In addition, they provide important insights into the potential use of host-associated,

behaviorally modifying compounds to increase the efficiency of parasitoids in the biological control of insect pests in storage environments.

Zusammenfassung

Zusammenfassung

Diese Doktorarbeit befasste sich mit der durch Semiochemikalien vermittelten Wirtssuche des Ektoparasitoiden *Holepyris sylvanidis* (Hymenoptera: Bethylidae), der die Larven verschiedener vorratsschädlicher Käfer parasitiert, aber die des amerikanischen Reismehlkäfers *Tribolium confusum* (Coleoptera: Tenebrionidae) bevorzugt. Vor dieser Arbeit war bereits bekannt, dass sich der Parasitoid bei der Wirtssuche anhand von Duftstoffen orientiert, die aus dem Larvalkot von *T. confusum* stammen, und dass die Attraktivität dieser wirtsspezifischen Kairomone für den Parasitoiden durch den Habitatduft (ausgehend von Futtersubstrat des Schädlings; hier Weizenvollkornschrot) verstärkt wird. Zudem dienen kutikuläre Kohlenwasserstoffe (KKW) der Wirtslarven als Kontaktkairomone zur Wirtserkennung und lösen Spurfolgeverhalten im Parasitoiden aus, aber nur über einen Zeitraum von zwei Tagen. Trotz dieser ersten Ergebnisse waren viele chemische und ökologische Aspekte der Wirtssuche von *H. sylvanidis* und das Potenzial wirtsspezifischer Kairomone zur Verbesserung der biologischen Schädlingsbekämpfung im Vorratsschutz weiterhin unbekannt.

Daher war es das Ziel dieser Arbeit, tiefere Einblicke in die chemisch vermittelte Wirtssuche von *H. sylvanidis* zu gewinnen. Verschiedene Verhaltensversuche und chemische Analysen von Wirtskairomonen mittels gekoppelter Gaschromatographie-Massenspektrometrie wurden durchgeführt, um die folgenden Fragen zu beantworten:

i) Welche Faktoren tragen dazu bei, dass die Aktivität der KKW-basierten Kairomone der Wirtsspuren nach drei Tagen verloren geht? (Kapitel 2)

ii) Unterscheiden die Weibchen von *H. sylvanidis* eine Wirtsart von einer Nichtwirtsart anhand eines wirtsspezifischen KKW-Musters auf der Kutikula? (Kapitel 3)

iii) Kann die zusätzliche Ausbringung von wirtsspezifischen Kairomonen das Wirtssucheverhalten des Parasitoiden fördern und somit zu einer effektiveren Bekämpfung von *T. confusum* führen? (Kapitel 4)

Im zweiten Kapitel untersuchten wir zuerst, ob die zeitlich begrenzte kairomonale Aktivität der Wirtsspuren möglicherweise auf durch Mikroorganismen verursachte Veränderungen in der Zusammensetzung des KKW-Profils zurückzuführen ist. Jedoch zeigten chemische Analysen, dass sich die KKW-Profile der Wirtsspuren weder qualitativ noch quantitativ in einem Zeitraum von zwei Tagen veränderten, unabhängig davon, ob diese unter sterilen und nicht-sterilen Bedingungen analysiert wurden. Diese Ergebnisse deuteten darauf hin, dass nicht der mikrobielle Abbau, sondern andere Faktoren für die temporäre, spurfolgeauslösende Aktivität der KKW verantwortlich sein könnten. Diese

Annahme wurde durch unsere Untersuchungen am Rasterelektronenmikroskop bestätigt. Innerhalb von zwei Tagen bildeten die Wirtspuren feste Mikrostrukturen, höchstwahrscheinlich aufgrund einer Selbstaggregation der KKW. Diese Prozesse waren jedoch durch die Zugabe von Hexan umkehrbar, sodass die kairomonale Aktivität von zwei Tage alten Wirtsspuren wiederhergestellt werden konnte. Der Parasitoid folgte diesen reaktivierten Wirtspuren im gleichen Maße, wie bei frisch gelegten Wirtsspuren. Basierend auf diesen Ergebnissen ist es sehr wahrscheinlich, dass die Verfestigung von langkettigen KKW in einer Wirtsspur die Wahrnehmbarkeit für *H. sylvanidis* im Laufe der Zeit so weit verringert, dass kein Spurfolgeverhalten mehr ausgelöst werden kann.

Im dritten Kapital untersuchten wir den Einfluss unterschiedlicher KKW-Profile von drei engverwandten Wirtsarten und einer entfernt verwandten Käferart auf das Wirtserkennungsverhalten von *H. sylvanidis*. In Verhaltensversuchen erkannten und akzeptierten weibliche Parasitoide die Larven der *Tribolium*-Arten als Wirte, während Larven von *Oryzaephilus surinamensis* abgelehnt wurden. Eine Veränderung im Verhalten des Parasitoiden konnten *O. surinamensis*-Larven nur auslösen, wenn lösungsmittelextrahierte Larven mit einem von *T. confusum*-Larven stammenden KKW-Extrakt behandelt wurden. Chemische Analysen der Larvenextrakten zeigten, dass sich die KKW-Profile der Wirtsarten und der Nicht-Wirtsart in ihrer Zusammensetzung unterschieden. Dies war vor allem auf die Fraktion der methylverzweigten Alkane (MeAL) zurückzuführen. Diese Verbindungen kamen auf der Kutikula von *Tribolium* spp. vor, fehlten aber bei *O. surinamensis*. Folglich dienen MeAL als Kontaktkairomone für die Wirtserkennung, anhand derer der Parasitoid eine Wirtsart von einer Nicht-Wirtsart unterscheidet.

Im vierten Kapitel untersuchten wir, ob flüchtige Duftstoffe, die entweder direkt mit *T. confusum*-Larven oder mit anderen Wirtsstadien (z.B. adulten Käfern) assoziiert sind, an der Wirtsfindung von *H. sylvanidis* aus größerer Entfernung beteiligt sind. Der Parasitoid wurde am stärksten von einem Mix aus zwei Schlüsselkomponenten aus dem Duftstoffbouquet des Larvenkots von *T. confusum* (z.B. (*E*)-2-nonenal und 1-Pentadecen, 2CM) in der niedrigsten Konzentration angelockt, während die höchste Konzentration abschreckend wirkte. Im Vergleich dazu waren zwei ausgewählte flüchtige Verbindungen, die von *T. confusum*-Adulten abgegeben werden, in allen Konzentrationen unattraktiv für den Parasitoiden. Anschließend untersuchten wir, ob die zusätzliche Ausbringung von 2CM (in der attraktivsten Konzentration) die Wirtssuche von *H. sylvanidis* verbessern kann. In dreitägigen Verhaltensversuchen wurden Parasitoiden *T. confusum*-Larven entweder mit zusätzlich ausgebrachten synthetischen Duftstoffen (= 2CM, Test) oder ohne (Kontrolle) angeboten. Im Vergleich zur Kontrolle entfernten Parasitoidweibchen in den Testkäfigen

innerhalb von 48h signifikant mehr Käferlarven aus der Ausgangspopulation und parasitierten bis zum Ende des Versuchs (nach 72h) eine signifikant größere Anzahl an Wirtslarven. Aus den Wirtlarven, die in Gegenwart von zusätzlich ausgebrachtem 2CM parasitiert wurden, schlüpften signifikant mehr Nachkommen von *H. sylvanidis*. Die Populationszunahme der F1 war jedoch auf einen signifikanten Anstieg männlicher Individuen zurückzuführen. Folglich können der Wirtsfindungserfolg und die Parasitierungsrate des Parasitoiden durch die Ausbringung synthetischer, wirtsassoziierter Kairomone verbessert werden, was aber mit Veränderungen in der Populations-zusammensetzung (Männchen/ Weibchen) der Nachkommen einhergeht.

Insgesamt tragen die in dieser Dissertation erzielten Ergebnisse zur durch Semiochemikalien vermittelten Wirtssuche eines natürlichen Gegenspielers von vorratsschädlichen Käfern zu einem besseren Verständnis der Interaktion zwischen Parasitoiden und ihren Wirtsarten bei. Darüber hinaus liefern sie wichtige Erkenntnisse über den potenziellen Einsatz von wirtsassoziierten, verhaltensverändernden Verbindungen zur Verbesserung der Wirksamkeit von Parasitoiden bei der biologischen Bekämpfung von Schadinsekten in Vorratslägern und in der verarbeitenden Lebensmittelindustrie.

Chapter 1:

General introduction and thesis outline

1.1. Current state of stored-product protection

After harvest, agricultural plant and food products are dried and stored to be available throughout the year. However, these products are permanently exposed to different environmental factors that can cause damage during transportation and storage. For instance, high humidity and temperature promote the growth of fungi and microorganisms, which can lead to mold growth and the production of mycotoxins (Schmidt et al. 2018; Fürstenau and Kroos 2020; Awuchi et al. 2021). In addition, biological factors such as birds, rodents, mice, and especially insects can provoke severe feeding damage and potential contaminations by dead and live individuals, body fragments, feces, and released chemicals (Hagstrum et al. 2010; Phillips and Throne 2010; Stejskal et al. 2015). Possible consequences are quantitative losses of stored products, reduction of quality (e.g., decreased baking ability of (cereal) flour), spoilage, and risks for human and animal health (Phillips and Throne 2010; Schmidt et al. 2018; Awuchi et al. 2021). Insects are responsible for 10% to 30% of food losses during storage and processing, estimated at 1.3 billion tons per year worldwide (Phillips and Throne 2010; FAO 2011; Pimentel 2011).

For almost a century, synthetic chemical pesticides have been widely used to control local pest populations effectively and rapidly in the warehousing and food processing industry (Fields and White 2002; Barratt et al. 2018). Although pesticides remain still the method of choice, especially when huge quantities of pest-infested food are discovered during the loading of rail and shipping containers (Hagstrum and Phillips 2017), the use of pesticides has been more and more questioned in recent decades. For example, the environmental fate of pesticides has been increasingly studied to assess potential toxic effects of chemical residues on consumers, workers, and the environment. This has led to stricter standards for the approval and banning of environmentally hazardous compounds such as methyl bromide (Phillips and Throne 2010; van Lenteren 2012; Barratt et al. 2018; Fürstenau and Kroos 2020). This substance was the most commonly used fumigant worldwide but was banned due to its ozone-depleting properties (Fields and White 2002). Furthermore, it has been increasingly reported in recent decades that local pest populations have evolved a reduced susceptibility to frequently used pesticides and, in some cases, have even become resistant. Due to resistance problems on the one hand and declining approval of active ingredients on the other hand, the range of available pesticides for pest management has decreased, making it more difficult to control stored-product pests (Zettler 1991; Niedermayer and Steidle 2013; Hagstrum and Phillips 2017; Riudavets 2018; Yao et al. 2019). Overall, knowledge about potential non-target effects on humans and the environment has led to an increased public demand to replace the use of conventional

pesticides with new and environmentally friendly pest control strategies (Schöller et al. 1997; Phillips and Throne 2010; van Lenteren 2012).

1.2. Integrated management of stored-product pests

Modern integrated management of stored-product pests (IPM) is an effective and sustainable approach that is based on comprehensive, current knowledge about the biology and technology of stored plant products as well as the biology of pest species and their interaction with the environment (e.g., natural enemies) (Schöller et al. 1997; Trematerra 1997; European Commission 2015; Hagstrum and Phillips 2017). Therefore, IPM is a decision support system that enables farmers and retailers to select appropriate control strategies with the least risk for humans and the environment. These control strategies may include non-chemical (e.g., biological, biotechnological, physical measures) and chemical measures, whereas the former are to be preferred (Eilenberg et al. 2001; Phillips and Throne 2010; European Commission 2015; Hagstrum and Phillips 2017). In addition, IPM integrates economic and ecological aspects by considering economic thresholds in decision-making processes. On this basis, chemically based control options should be only taken into consideration in situations when a pest population becomes an economic threat, and the value of potential losses exceeds the costs of applied control measures (Trematerra 1997; Stejskal 2003; European Commission 2015; Hagstrum and Phillips 2017). Overall, IPM is an integral part of the "farm to fork" strategy of the European Green Deals in which the use of chemical synthetic pesticides should be reduced by 50% by 2030 and alternative and non-chemical measures are increasingly promoted (European Commission 2022).

IPM in storage facilities and food processing industry is a three-tier approach based on prevention, early detection, and control (NAP - National Action Plan on Sustainable Use of Plant Protection Products 2019). Firstly, hygienic and sanitation programs (e.g., the cleanup of spilled products), improved building designs and an adequate temperature regime (e.g., grain aeration) are important methods to prevent insects from entering warehouses and infesting stored and processed commodities (Schöller et al. 1997; Phillips and Throne 2010). Secondly, sampling (e.g., pheromone-baited insect traps and grain trier) and monitoring environmental factors (e.g., relative humidity and temperature in grain masses) are of great importance for early detection of pest species and surveillance of their population dynamics. In addition, new technological methods (e.g., acoustic techniques, approaches using real-time Polymerase Chain Reaction (PCR) or digital X-ray equipment) could be used as further detection methods in the future (Trematerra 2012; Solà Cassi 2017; Hagstrum and Phillips 2017; Müller-Blenkle et al. 2018). Lastly, if an infestation needs to be

controlled, physical (e.g., irradiation and controlled and modified atmosphere), biological (e.g., release of entomophagous insects) and other non-chemical control options should be preferentially applied. Only in cases that infestations exceed the economic threshold, chemical control options (e.g., the conventional pesticides) are allowed as a last resort but are excluded from use in organic farming and mills (Phillips and Throne 2010; European Commission 2015; Hagstrum and Phillips 2017; Fürstenau and Kroos 2020).

1.3. Biological control of stored-product pests

As part of IPM approaches, biologically based control methods have become increasingly important in agriculture in recent decades. This is because they are known to be more target-specific, having a low risk of chemical residues and thus, have fewer side effects on the environment and humans. In addition, these methods have been proven to be a technically and economically feasible alternative to chemical pest control (Cox 2004; Delfosse 2005; van Lenteren 2012; Riudavets 2018; van Lenteren et al. 2018).

For IPM approaches in storage facilities and the food processing industry, the use of behaviorally active volatiles and natural enemies (e.g., parasitoids) are two common biologically based control strategies (Schöller et al. 1997; Flinn and Schöller 2012; Trematerra 2012; Fürstenau and Kroos 2020). For instance, the pheromones of more than 30 different pest beetles and moths are already commercially available as slow-release attractants and are used primarily to monitor local pest populations, but also for other alternative pest management strategies such as mass trapping and mating disruption (Sword and Van Ryckeghem 2010a, b; Phillips and Throne 2010; Trematerra 2012). Parasitoids are mainly used in augmentative biological control approaches. For this purpose, large numbers of mass-reared individuals are released either to obtain an immediate control by applied parasitoids (inundative biological control) or to propagate in order to control a pest population over a long period (inoculative biological control) (Eilenberg et al. 2001; Flinn and Schöller 2012). Furthermore, storage facilities provide environments that seem to represent favorable locations for the application of parasitoids because the building structures may prevent parasitoids from leaving (Flinn and Schöller 2012).

However, the potential of parasitoids as alternative control tools for protection of stored products is only partially exploited (van Lenteren 2012; van Lenteren et al. 2018; Fürstenau and Kroos 2020). In many cases, the standard of pest management is still chemically based. The main reason is due to the fact that chemically based control methods often have higher efficacy at low production costs than biologically based ones (Schöller

1998; van Lenteren 2012; van Lenteren et al. 2018). The efficacy gap between biologically and chemically based control measures may discourage farmers and retailers from implementing biological control strategies (Fürstenau and Kroos 2020).

For an improved performance of parasitoids as biological control agent, an in-depth knowledge about the biology and the chemically mediated host-searching behavior of parasitoids, the interactions between parasitoids and their respective host species as well as the role of semiochemicals involved, is needed to accurately determine how, when, and to what extent parasitoids can be released to control pest insects of stored products (Schöller et al. 1997; Fürstenau and Kroos 2020).

1.4. Chemically mediated host search of parasitoids

Living organisms are known to communicate with their environment by receiving and emitting information. Semiochemicals (i.e., both volatile organic compounds with low to medium molecular weight and less volatile contact chemicals) can convey information between organisms of the same or different trophic levels and alter the behavior of the organisms involved (Law and Regnier 1971; Nordlund and Lewis 1976; Fürstenau and Kroos 2020). These substances can be classified as pheromones when mediating intraspecific communication, or as allelochemicals when mediating the interaction between different species (interspecific interactions). The latter group of semiochemicals is further divided into allomones, kairomones, and synomones depending on whether the perception of a particular signal benefits the emitter (allomones), the receiver (kairomones) or both (synomones). In some cases, however, compounds can be assigned to different classes, e.g., when the pheromone of one species evokes a behavioral response in a receiving organism of a different species (Mori 2010; Wehrenfennig et al. 2013).

In a highly dynamic environment foraging parasitoid females often face the challenging task to optimize their host search in order to locate potential hosts that represent a suitable food source for them and their progeny. Therefore, the host-finding success is directly linked to the reproductive success of parasitoid females (Vet and Dicke 1992; Fatouros et al. 2008). Parasitoids may use numerous stimuli such as visual, chemical, acoustic, and gustatory cues for host location. Semiochemicals, and in particular kairomones may provide reliable information about the presence of a potential host, especially when the host is hardly visible through hiding (Steidle and Van Loon 2003; Fatouros et al. 2008; Aartsma et al. 2019). Therefore, the perception of host-specific kairomones may become crucial for successful foraging behavior of parasitoids. It is known that parasitoids can eavesdrop on the intraspecific communication of host species and use,

for example, sex and aggregation pheromones for host location. In addition, parasitoids can respond to volatiles directly associated to the host (e.g., fecal volatiles or compounds on the host cuticle) as well as to indirectly associated ones (e.g., volatiles from the host plant or feeding substrate) (Vet and Dicke 1992; Steidle and Van Loon 2003). Indirectly host-associated cues comprise, for example, volatiles released by plants in response to herbivore feeding and oviposition (herbivore- or oviposition-induced plant volatiles, HIPVs and OIPVs) as well as compounds which are released by host-associated organisms such as mites (Ruther and Steidle 2000; Steidle et al. 2003; Hilker and Meiners 2011; Turlings and Erb 2018).

For the different stages of the host search (= host habitat location, host location, host recognition and host acceptance), parasitoids are known to exploit various compounds as kairomones, which may thus regulate a fine-tuned host finding behavior (Vinson 1998; Fatouros et al. 2008). Highly volatile long-range cues (e.g., HIPVs, OIPVs, host's sex pheromones) are initially important to guide foraging parasitoids to the host habitat and to spot the host location (Vet and Dicke 1992; Vinson 1998; Steidle and Van Loon 2003). Upon arrival, parasitoids exploit less volatile compounds, i.e. short-range cues (e.g., cuticular hydrocarbons) to find and recognize potential hosts in close vicinity (Vinson 1998; Howard and Blomquist 2005; Colazza et al. 2014).

1.4.1. Cuticular hydrocarbons (CHCs)

Cuticular hydrocarbons (CHCs) are the most abundant lipid class on the cuticle of all insects that fulfill several important functions. CHCs play a central role in protecting insects from various environmental stresses (e.g., water loss, radiation, pathogens) as well as in mediating chemical communication in social and solitary insects (Howard and Blomquist 2005; Otte et al. 2018; Blomquist and Ginzel 2021). The multifunctionality of the epicuticular layer mainly results from the physical traits of the CHCs present (Gibbs 1998; Menzel et al. 2019). In general, CHCs are a highly diverse group of chemicals that includes linear and methyl-branched, saturated, and unsaturated hydrocarbons (Blomquist and Bagnères 2010). CHCs also differ in their melting temperatures due to differences in the chain length and structural features (e.g., double bonds, methyl branches) (Gibbs and Pomonis 1995; Gibbs and Rajpurohit 2010). Due to the different physical properties of CHCs, the epicuticular layer of hydrocarbons may form a solid-liquid mixture over a wide temperature range, providing both waterproofing and signaling under fluctuating environmental conditions (Gibbs 2002; Sprenger et al. 2018; Menzel et al. 2019).

In many insect species, CHCs serve as contact pheromones and kairomones for recognition of gender, caste, nest mates, mutualistic partners, and hosts (Howard and Blomquist 2005). The CHC composition can range from very simple blends with a few compounds to highly complex multi-component blends with up to 100 compounds, forming different CHC patterns. These patterns do not only vary among species, but also between different developmental stages, ages, and the sexes of a species (Blomquist and Bagnères 2010; Pokorny and Ruther 2023). However, many studies have already indicated that the intra- and interspecific chemical communication generally relies on a small number of components. For some species, individual CHCs are sufficient to elicit a specific behavior, whereas in others, the combination of several compounds is essential (Sugeno et al. 2006; Colazza et al. 2009; Silk et al. 2011; Würf et al. 2020; Ayelo et al. 2022). In particular, methyl-branched alkanes are of great importance for triggering a behavioral response in insects in the search for (nest) mates or mutualistic partners (Spikes et al. 2010; Silk et al. 2011; Ablard et al. 2012; Kühbandner et al. 2012b; Bello et al. 2015; De Narbonne et al. 2016; Sakata et al. 2017).

While walking on the plant surface or feeding substrate, insects may leave unintentionally chemical trails, which consist mainly of CHCs. Some parasitoid species are known to respond to these chemical stimuli during host search (Colazza et al. 2014). These parasitoids remain in close contact with CHC trails by frequent antennation and follow them in zigzag movements to detect a potential host nearby (Howard and Flinn 1990; Howard et al. 1998). Trail-following behavior has been previously described for parasitoid species belonging to the Bethylidae (Howard and Flinn 1990; Fürstenau and Hilker 2017), Braconidae (Rostás and Wölfling 2009), and Scelionidae (Colazza et al. 2009; Salerno et al. 2009; Gomes Lagôa et al. 2020). For these species, CHC trails laid onto the substrate may serve as contact kairomones for host location, host recognition, and host sex discrimination. Furthermore, CHC trails can be used to find mating partners or for predator avoidance (Nakashima et al. 2004; Bernal and Luck 2007).

1.5. The application of semiochemicals in agricultural systems

According to van Lenteren et al. (2018), augmentative biological control has been successfully applied on 30 million hectares worldwide in 2015 to control pest populations. Despite this success in implementing biological control strategies in pest management, the potential of natural enemies (here parasitoids) is far from being fully exploited (van Lenteren 2012; van Lenteren et al. 2018). For instance, the host search of released parasitoids may be affected by complex habitat structures, resulting in a less effective control of pest

Table 1 Examples for the field application of semiochemicals in different agricultural systems

Semiochemicals		Biological control strategy[b]	Parasitoid species	Pest (herbivorous) species	Field crop species	Behavioral effects on parasitoids[c]	References
Type[a]	Compound						
HIPV	α-farnesene	ABC	*Anaphes iole*	*Lygus lineolaris*	Cotton	A, PR	Williams et al. (2008)
HIPV	(*Z*)-3-hexenyl acetate	ABC	*Anaphes iole*	*Lygus lineolaris*	Cotton	A, PR	Williams et al. (2008)
HIPV	4′-ethyl-acetophenone	ABC	*Peristenus spretus*	Apolygus lucorum	Cotton	A, PR	Xiu et al. (2019)
HIPV	*cis*-jasmone	CBC	*Trissolcus podisi*	*Euschistus heros*	Soybean	A	Vieira et al. (2013)
HIPV	*m*-cymene	ABC	*Peristenus spretus*	*Apolygus lucorum*	Cotton	A, PR	Xiu et al. (2019)
HIPV	MeSA[d]	CBC	*Pseudopraon sp.*	*Halyomorpha halys*	Alberta spruce	A, PR	Lee et al. (2022)
HIPV	MeSA[d]	CBC	*Diadegma semiclausum*	*Plutella xylostella*	Turnip	A	Orre et al. (2010)
HIPVs	Mix of MeSA[d], benzaldehyde + *cis*-3-hexenyl (10:3:3)	ABC	*Trichogramma dendrolini*	*Grapholita molesta*	Peach orchard	A	Zhao et al. (2022)
HIPVs	mix of MeSA[d], benzaldehyde + linalool oxide (10:3:3)	ABC	*Trichogramma dendrolini*	*Grapholita molesta*	Peach orchard	A	Zhao et al. (2022)
HIPV	Ocimene	CBC	*Thinodytes cephalon*	*Ophiomyia simplex*	Asparagus	A, PR	Ingrao et al. (2019)
HIPVs	Mix of (*Z*)-3-hexenyl acetate, *n*-heptanal, α-pinene + sabinene	ABC	*Cotesia vestalis*	*Plutella xylostella*	Turnip	A, PR	Uefune et al. (2012)
PH	Jasmonic acid	CBC	*Anagrus nilaparvatae*	*Nilaparvata lugens*	Rice	A, PR	Lou et al. (2005)
SP	Cyclolavandulyl butyrate	CBC	*Anagyrus subalbipes, A. sawadai*	*Planococcus kraunhiae*	Different orchards	A	Sugawara and Ueno (2020)
SP (female)	(+)-(4a*S*,7*S*,7a*R*)-nepetalactone	CBC	*Praon valucre*	*Sitobion avenae*	Winter barley	A, PR	Glinwood et al. (1998)
			Aphidus rhopalosiphi			A, PR	
SP (female)	mix of hexadecanal, (*Z*)-7-hexadecenal, (*Z*)-9-hexadecenal + (*Z*)-11-hexadecenal	ABC	*Trichogramma pretiosum*	*Helicoverpa zea*	Cow pea	A, PR	Lewis et al. (1982)
		CBC	*Trichogramma sp.*		Cotton	A, PR	
SP (male)	(*E*)-2-hexanal	CBC	*Trissolcus sp.*	*Euschistus heros*	Soybean	A, PR	Vieira et al. (2014)

[a] HIPV = herbivore induced plant volatile, PH = phytohormone, SP = sex pheromone, [b] ABC = augmentative biological control, CBC = conservation biological control, [c] A = increased attraction, PR = increased parasitism rate; [d] MeSA = methyl salicylate

populations under field conditions (Meiners and Peri 2013; Zhao et al. 2022). A promising approach to improve the use of parasitoids in biological control is the additional application of synthetic semiochemicals. This involves the use of behavior-modifying substances to attract and retain parasitoids to a specific area, thereby, increasing their performance as biological control agents of pest species (Cox 2004; Phillips and Throne 2010; Kaplan 2012; Meiners and Peri 2013; Fürstenau and Kroos 2020; Ayelo et al. 2021).

The effectiveness of long-range attractants on the foraging behavior of parasitoids has already been demonstrated under crop field conditions (Table 1). In these studies, semiochemicals were used for either augmentative biological control (ABC) or conservational biological control (CBC). In contrast to ABC, CBC approaches modify the habitat to protect naturally occurring parasitoids and stimulate their performance in controlling pest populations instead of introducing new ones. In this case, semiochemicals recruit parasitoids from the adjacent fields to achieve their appropriate temporal-spatial distribution in pest control. The used volatiles are either herbivore-induced plant volatiles (HIPVs), plant hormones, or components of the sex pheromone of target pest species. In some studies, single compounds were sufficient to alter the parasitoid's behavior, whereas in other studies, a multi-component blend was necessary. Overall, the application of synthetic attractants led to an increase in the abundance of parasitoids and parasitism rate in test fields (Table 1).

To date, two shikimic acid pathway-derived HIPVs, methyl salicylate (MeSA) and 2-phenylethanol, have been used as active ingredients of two commercially available attractants (MeSA: PredaLure, AgBio Inc., Westminster, CO, USA; 2-phenylethanol: Benallure MSRTS Technologies, Ames, IA, USA) (Braasch et al. 2012). Particularly, the use of PredaLure appears to be a promising approach for conservation biological control. Several studies have already shown that application of PredaLure in the field increased the abundance of various predators (e.g., Chrysopidae, Coccinellidae, Syrphidae), resulting in an improved predation on local pest populations (Rodriguez-Saona et al. 2011; Salamanca et al. 2019; Lee et al. 2022). In addition, an enhancing effect of PredaLure on the parasitization behavior of *Pseudopraon* species (Hymenoptera: Braconidae), antagonists of the brown marmorated stinkbug *Halyomorpha halys* (Hemiptera: Pentatomidae), had been recently reported by a study of Lee et al. (2022).

The use of synthetic volatiles with long-range behavioral activity on parasitoids may also offer new prospectives for biologically based control approaches of stored-product pest species (Fürstenau and Kroos 2020). Host-associated volatiles could be used as lures in traps for monitoring population dynamics of naturally occurring and released parasitoids in storage facilities. This might help optimizing the timing of parasitoid release and later

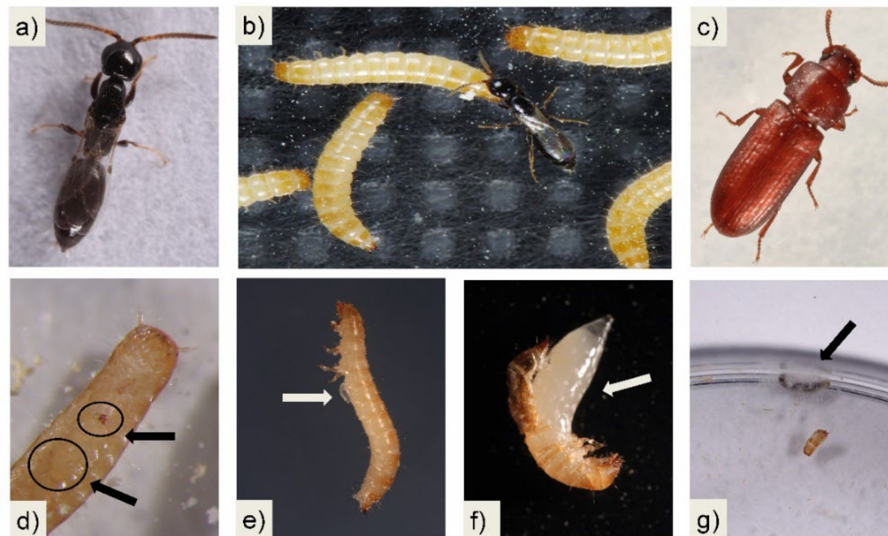

Figure 1 *Holepyris sylvanidis* and *Tribolium confusum*.
a) *H. sylvanidis* female, **b)** interaction of a *H. sylvanidis* female and a *T. confusum* larva, **c)** *T. confusum* adult, **d)** *T. confusum* larva with host feeding marks, **e)** *H. sylvanidis* egg on a paralyzed *T. confusum* larva, **f)** destructive feeding of *H. sylvanidis* larva on a *T. confusum* larva, **g)** a *H. sylvanidis* pupa in a cocoon. The arrows in the photos indicate the position of the host feeding marks (d), parasitoid egg (e), larva (f), and pupa (g)

removal from the storage environment prior to postharvest processing of plant products (Schöller 1998; Trematerra 2012). Furthermore, the additional release of synthetic kairomones may modify the host-searching behavior of parasitoids, for instance by intensifying the attraction of parasitoids to infestation sites (Cox 2004; Phillips and Throne 2010). This may improve the parasitoid's host-searching activity and parasitization success, boosting their effectiveness in controlling pest populations (Cox 2004; Williams et al. 2008; Phillips and Throne 2010; Vieira et al. 2014).

Despite the potential of host-associated semiochemicals to improve biological control in stored-product protection (e.g., the use of parasitoid), chemically mediated host search behavior has been only studied in three bethylid and two pteromalid species (Howard and Flinn 1990; Howard et al. 1998; Ruther and Steidle 2000; Steidle et al. 2001a, b; Steiner et al. 2007; Fürstenau et al. 2016; Fürstenau and Hilker 2017; Giunti et al. 2021). Therefore, knowledge about the chemistry of compounds (= kairomones) used by foraging parasitoids for host recognition and their potential to enhance parasitoid efficiency in controlling stored product pests is still limited.

1.6. The study system

To fill the aforementioned gaps in knowledge, this dissertation thesis focused on the semiochemicals mediating host search of the larval ectoparasitoid *Holepyris sylvanidis* (Brèthes 1913) (Hymenoptera: Bethylidae) (Figure 1a). This parasitoid is described as a

cosmopolitan parasitoid species (Evans 1969) but so far, its occurrence in storage facilities and mills has been reported only from Bangladesh, Egypt, Germany, and Greece (Awadallah et al. 1985; Ahmed and Islam 1988; Prozell and Schöller 1998; Eliopoulos et al. 2002). The potential host range of *H. sylvanidis* includes different stored-product pest beetles belonging to the genera of Cucujidae, Tenebrionidae, and Silvanidae (Evans 1969; Hagstrum and Subramanyam 2009; Amante et al. 2017b). However, only the interaction between the parasitoid and its preferred host species, larvae of the confused flour beetle, *Tribolium confusum* (Du Val 1863) (Coleoptera: Tenebrionidae) had been investigated in detail prior to this thesis (Figure 1b + c) (Ahmed and Islam 1988; Ahmed et al. 1997; Fürstenau et al. 2016; Amante et al. 2017a, 2018; Fürstenau and Hilker 2017).

Holepyris sylvanidis females destructively feed on larvae of *T. confusum*, mainly on the younger ones (1st - 3rd instars) that consequently die and are not used for oviposition (Figure 1d). Host feeding is essential for synovigenic parasitoid females since it provides nutrients for egg maturation and promotes their longevity (Jervis and Kidd 1986; Ahmed and Islam 1988; Ahmed et al. 1997; Amante et al. 2017a). In rare cases, feeding marks on host larvae were also observed prior to egg deposition (own observation). Older *T. confusum* larvae (4th and 5th instars) are used for oviposition because the development of parasitoid offspring depends on available nutrients supplied by the host (Figure 1e) (Ahmed et al. 1997; Vinson 1998; Amante et al. 2017a). Therefore, the selection of suitable hosts is critical for the survival of parasitoid progeny (Vinson 1998).

When a *H. sylvanidis* female encounters a *T. confusum* larva, it intensively examines the cuticle of the potential host with its antennae (Figure 2a). If the parasitoid recognizes the larva as a suitable host, it immediately attacks. Therefore, the parasitoid bends its body around the host larva to inject a venom in the thoracic region. In the beginning, the host larva attempts to shake off the parasitoid female, resulting in a repeated rotation of the parasitoid female around the host larva's body (Figure 2b + c). Within seconds, the host larva becomes permanently paralyzed and motionless due to the venom injected. After successful attack, the parasitoid female surveys again the paralyzed host larva with its antennae (Figure 2d). Then, it grabs one antenna of the host larva with its mandibles and removes the host larva to a hiding place where the oviposition can take place (Figure 2e + f) (Abdella et al. 1985; Ahmed et al. 1997; Amante et al. 2017a). It is not yet known why *H. sylvanidis* dislocates paralyzed host larvae for egg deposition. One possible explanation could be that populations of *T. confusum* are known to be cannibalistic in situations with a high density of individuals and an increasing shortage of food resources. In particular, immobile developmental stages such as eggs and pupae are preyed by adults (Benoit et al. 1998; Giray et al. 2001). Another explanation could be that females of other bethylid

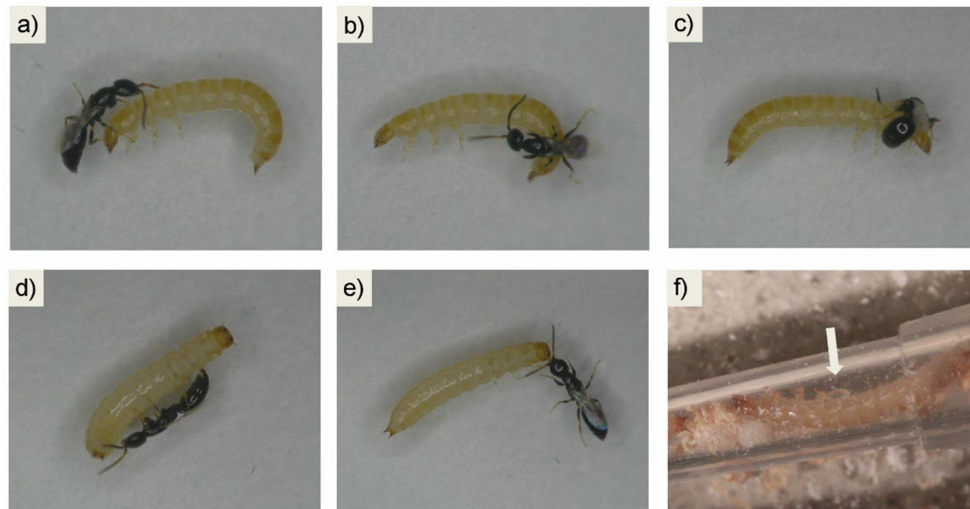

Figure 2 Host recognition behavior of *Holepyris sylvanidis*
a) antennation on a potential host larva for host recognition (here 4th instar of *T. confusum*) by a *H. sylvanidis* female, **b)** + **c)** paralyzation of host larva indicated by the bended abdomen of the parasitoid female, **d)** antennation on the paralyzed host larva, **e)** removal of the paralyzed host larva to a safe shelter, **f)** parasitized host larva in a pipette tip provided as shelter. The arrow indicates the position of the parasitoid egg

species (e.g., *Cephalonomia tarsalis*) are known to show agonistic and competitive behavior by interfering with each other's foraging and reproductive behavior (Eliopoulos et al. 2017; Amante et al. 2017b). In order to avoid mutual intraspecific interference by conspecific females and future attacks by *T. confusum*, *H. sylvanidis* may hide paralyzed host larvae to make them harder to detect and easier to defend. This may lead to an increased reproductive success of parasitoid females (= oviposition).

Holepyris sylvanidis is an arrhenotokous parasitoid species in which male offspring emerge from unfertilized eggs and female offspring from fertilized eggs (Abdella et al. 1985). Furthermore, mating can enhance the egg laying activity of parasitoid females (Amante et al. 2017a). For oviposition, the female places one egg longitudinally between the first and second abdominal sternite of a paralyzed host (Figure 1e). Approximately two days after oviposition, the parasitoid larva hatches and immediately starts to externally feed on the host until, after nine days, the empty exoskeleton of the host remains (Figure 1f + g). Then the parasitoid larva spins a whitish cocoon within two days prior to pupation (Figure 1g). After approximately two weeks, the first males emerge and a few days later the females (Ahmed and Islam 1988; Ahmed et al. 1997; Amante et al. 2017a). Overall, the development from parasitoid egg to adult takes about four weeks in the permanent rearing of *H. sylvanidis* in the laboratory of the Julius Kühn-Institute (JKI, Berlin, Germany; abiotic conditions: 25 ± 1°C and 65 ± 5% relative humidity in permanent darkness).

The host species *T. confusum* is one of the major pests of stored grain and grain products (e.g., flour, pasta; Figure 1c), but also infests a broad range of other plant products

in storage facilities and food processing industry (Good 1933; Hagstrum and Subramanyam 2009). Due to a high reproductive rate and long reproductive period of female beetles, *T. confusum* exhibits a high growth rate, resulting in a rapidly increasing population size. Moreover, *T. confusum* may have a relatively short development time, depending on diet, temperature, and humidity (Brindley 1930; Good 1933; Weidner 1983; Reichmuth et al. 2007). In the laboratory at the JKI the development from eggs to adults takes between 49 to 63 days when the beetle is reared on wheat grist at 25 ± 1°C, 65 ± 5% relative humidity and permanent darkness. Under these conditions, we observed up to five larval instar levels of *T. confusum*.

In addition to quantitative losses of stored products due to feeding by *T. confusum* adults and larvae, the release of benzoquinones (methyl- and ethyl-*p*-benzoquinone) by the beetle is also a major problem (Weidner 1983; Reichmuth et al. 2007). These compounds are produced by adult beetles in two pairs of exocrine glands, the prothoracic glands and the pygidial glands; the benzoquinones are components of the defensive secretion that is released in response to overpopulation and food shortages (Markarian et al. 1978; Faustini and Burkholder 1987). Characteristic of infested flour is a pink color and an unpleasant smell. Since benzoquinones are known to be carcinogenic to humans and animals, the infested flour must be completely destroyed (Reichmuth et al. 2007; Lis et al. 2011). In recent decades, it has been frequently reported that local populations of *T. confusum* have become less sensitive to commonly used insecticides and, in some cases, were even resistant (Zettler 1991; Hagstrum and Phillips 2017; Yao et al. 2019). Therefore, there is an urgent need for alternative non-synthetic chemical control measures. The release of *H. sylvanidis* as a natural antagonist represents a promising option for biological pest control of *T. confusum.*

1.7. Research objectives and hypotheses

The semiochemicals mediating host-searching behavior of *H. sylvanidis* has been already investigated in previous studies by Fürstenau et al. (2016) and Fürstenau and Hilker (2017). On this basis, the parasitoid is known to be guided by host-specific volatiles released from larval feces of *T. confusum* for long-range host location. Two key larval kairomonal compounds, (*E*)-2-nonenal and 1-pentadecene, have already been identified prior to this thesis. Interestingly, the attractiveness of both compounds for foraging parasitoids was significantly increased by the addition of habitat volatiles from the feeding substrate (here wheat grist), indicating the importance of background odor during host search of the parasitoid (Fürstenau et al. 2016). In addition, it has been shown that CHCs from

T. confusum larvae act as contact kairomones, mediating trail-following and host recognition behavior in *H. sylvanidis* (Fürstenau and Hilker 2017).

Despite these initial findings, many questions regarding host-searching behavior of the parasitoid remained unanswered. For example, Fürstenau and Hilker (2017) have revealed that the kairomonal activity of *T. confusum* larval trails lasted only for a maximum of three days after deposition even though the host trails consisted of long-chain, low volatile CHCs. The phenomenon that host CHC trails lose their kairomonal activity over time has also been described in other parasitoid-host systems (Howard and Flinn 1990; Howard et al. 1998; Rostás and Wölfling 2009). The effects of kairomonal loss of CHC trails on host-searching behavior of parasitoids have previously been studied, whereas the factors driving are still unknown. The hypothesis that host- or host-habitat-associated microorganisms might be responsible for the degradation of CHCs present in host trails was tested in this thesis.

In addition, prior to this thesis, *H. sylvanidis* was described in the literature only as a polyphagous antagonist of various warehouse beetle species, while detailed information on the host range of the parasitoid was limited. Therefore, we wanted to know whether parasitoid females recognize and accept larvae of other beetle species than *T. confusum* as hosts. We also addressed the question of whether host recognition behavior of *H. sylvanidis* is based on the perception of a specific group of compounds present on the cuticle of different host species. We studied the hypothesis that host species of *H. sylvanidis* share a common pattern of CHCs that serves as a host recognition cue.

Lastly, we asked whether volatiles associated to other development stages of *T. confusum* than larvae (e.g., beetle adults) are exploited by *H. sylvanidis* for (long-range) host location. Moreover, we investigated the effect of additionally applied, synthetic kairomones on the host-searching behavior of the parasitoid and on the population dynamics of its progeny. This allowed us to test the hypothesis that the presence of long-range attractants improves the effectiveness of *H. sylvanidis* as a biological control agent of *T. confusum*. Overall, this doctoral thesis focused on the host finding (Figure 3a), the host recognition (Figure 3b), and the host search efficiency (Figure 3c) of *H. sylvanidis.*

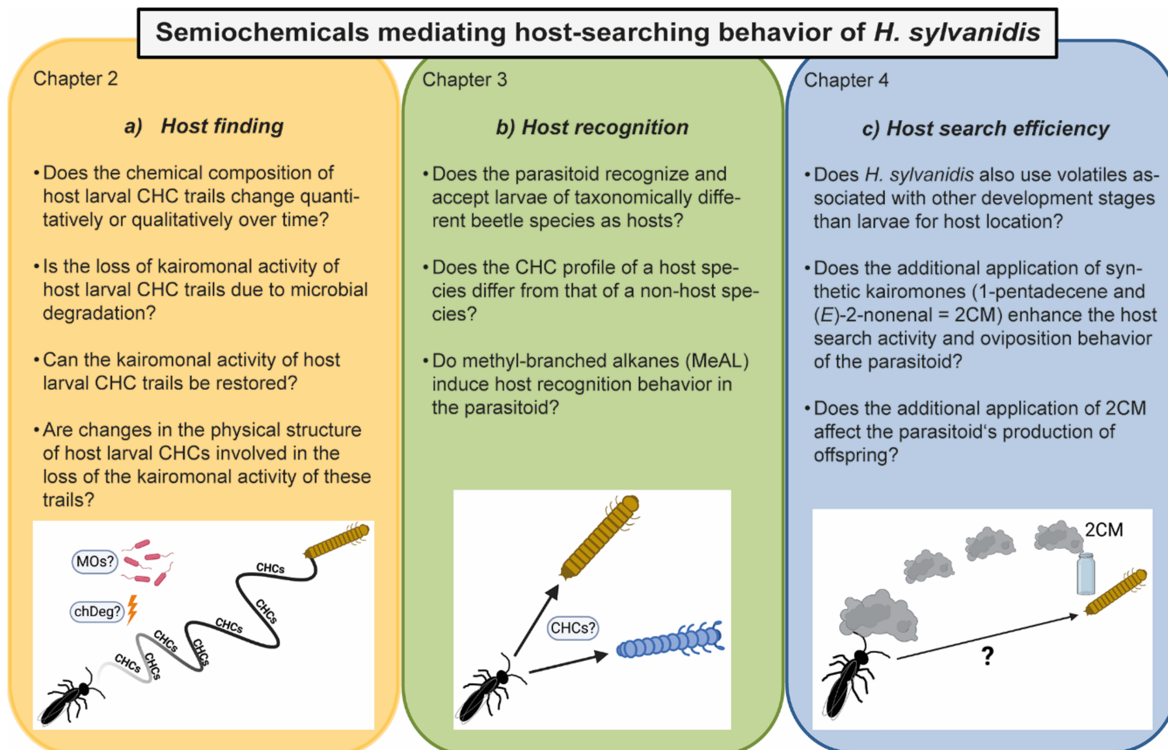

Figure 3 Overview of the main research questions addressed in this doctoral thesis on the semiochemicals mediating host-searching behavior of *Holepyris sylvanidis* regarding **a)** host finding (Chapter 2), **b)** host recognition (Chapter 3), and **c)** host search efficiency (Chapter 4). Abbreviations: CHC(s) = cuticular hydrocarbon(s), MOs = microorganisms, chDeg = chemical degradation processes, MeAL = methyl-branched alkanes, 2CM = two component mix containing 1-pentadecene and (*E*)-2-nonenal, ratio 1:1). Figure was created with BioRender.com

In **Chapter 2** we investigated potential factors driving the loss of kairomonal activity of larval CHC trails of *T. confusum* over time. To test the hypothesis that the trails are degraded by microbes associated with the host or its environment, we first chemically analyzed trails consisting of hexane extracts of *T. confusum* larvae 0, 24, and 48 h after deposition under sterile and non-sterile conditions. Using gas chromatography-mass spectrometry (GC-MS) analysis, we compared the qualitative and quantitative composition of CHC trails between the different time intervals and investigated the possible microbial contribution to the activity loss of CHC trails. In trail-following bioassays, we examined the behavioral responses of *H. sylvanidis* females (i) to CHC trails at different time intervals after deposition and (ii) to CHC trails 0 and 48 h after deposition which had been treated with hexane before being offered to parasitoids. The latter treatment enabled us to study whether the kairomonal activity of CHC trails could be restored by applying hexane as solvent. In addition, we studied whether the physical state of CHCs determines the kairomonal activity of CHC trails over time. We used cryo-scanning electron microscopy (cryo-SEM) to analyze the structural changes of CHC trails in the course of time.

In **Chapter 3** we addressed the question of whether *H. sylvanidis* females recognize their host species by a specific CHC pattern on the cuticle that is common to the different

host species. In contact bioassays, we studied the host finding and host recognition behavior of parasitoid females by offering live and dead larvae of four different beetle species (*Tribolium castaneum*, *T. confusum*, *T. destructor*, *Oryzaephilus surinamensis*) as potential hosts. In a subsequent non-choice bioassay, we wanted to know whether beetle species, which had been successfully recognized as hosts, are also accepted for oviposition. Therefore, we quantified the number of parasitoid offspring emerged from parasitized host larvae per beetle species. To figure out to what extent the CHC profile of a host species differs from a non-host species, we chemically analyzed crude larval hexane extracts of *Tribolium* spp. and *O. surinamensis* by GC-MS. Based on the results of bioassays and GC-MS analyses, we selected *T. confusum* and *O. surinamensis* as host and non-host species, respectively, for further contact bioassays. First, we examined whether CHCs present on the cuticle of the host species (*T. confusum*) serve as contact kairomones for host recognition. To this end, we applied crude larval extracts of *T. confusum* onto dead, extracted larvae of the non-host species, *O. surinamensis,* to test whether treated larvae of the latter could elicit host recognition behavior in *H. sylvanidis*. Furthermore, we analyzed which substance group within the CHC profile of *T. confusum* might be responsible for host recognition in *H. sylvanidis*. Therefore, we fractionated crude larval extracts of *T. confusum* before examining the behavioral responses of parasitoid females to dead, extracted host larvae treated with different fractions.

In **Chapter 4** the question whether the additional application of (long-range) kairomones can improve the host-searching activity and host-finding success of *H. sylvanidis* was studied. In Y-tube olfactometer bioassays, we analyzed the attraction of directly and indirectly host-associated volatiles to parasitoid females. We tested different concentrations of a mix of the two key larval kairomonal compounds, (*E*)-2-nonenal and 1-pentadecene (2CM). Additionally, we investigated the behavioral response of *H. sylvanidis* to two compounds, methyl-*p*-benzoquinone and 4,8-dimethyldecanal, emitted by *T. confusum* adults. As *H. sylvanidis* was strongly attracted by 2CM in the bioassays, we analyzed the release kinetics of (*E*)-2-nonenal and 1-pentadecene at different concentrations. Furthermore, behavioral tests in flight cages were performed to elucidate how the presence of additionally applied 2CM affects the host-searching behavior of *H. sylvanidis*. At different time intervals (1, 24, 48, and 72 h after parasitoid release) we counted the number of *T. confusum* larvae which had been removed by the parasitoid. At the end of the experiment (72°h), the number of parasitized *T. confusum* larvae as well as the number and sex of parasitoid offspring emerging from the host larvae were recorded. Therefore, we could assess possible effects of host-associated kairomones (here 2CM) not

only on the host search efficiency of *H. sylvanidis* but also on the population composition of the parasitoid in the next generation (F1).

In **Chapter 5** of this thesis, these three studies (Chapter 2-4) are discussed by taking an overarching perspective. To this end, I compared the obtained results with the current state of knowledge on HIPVs, which serve as semiochemicals for host-searching parasitoids and are already used as long-range attractants in practice in the field. In addition, other aspects of host search of parasitoids are addressed that are not covered in the previous chapters. These include possible effects of different host diets on the CHC composition and their function as host-indicating cues, as well as the use of host-specific CHCs as general cues for oligophagous and polyphagous parasitoids. Finally, the potential of host-associated semiochemicals to enhance parasitoid performance in biological control approaches is discussed.

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Chapter 2:

Cuticular hydrocarbon trails released by host larvae lose their kairomonal activity for parasitoids by solidification

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Cuticular Hydrocarbon Trails Released by Host Larvae Lose their Kairomonal Activity for Parasitoids by Solidification

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Abstract

Successful host search by parasitic wasps is often mediated by host-associated chemical cues. The ectoparasitoid *Holepyris sylvanidis* is known to follow chemical trails released by host larvae of the confused flour beetle, *Tribolium confusum*, for short-range host location. Although the hexane-extractable trails consist of stable, long-chain cuticular hydrocarbons (CHCs) with low volatility, the kairomonal activity of a trail is lost two days after release. Here, we studied whether this loss of kairomonal activity is due to changes in the chemical trail composition induced by microbial activity. We chemically analyzed trails consisting of hexane extracts of *T. confusum* larvae after different time intervals past deposition under sterile and non-sterile conditions. GC-MS analyses revealed that the qualitative and quantitative pattern of the long-chain CHCs of larval trails did not significantly change over time, neither under non-sterile nor sterile conditions. Hence, our results show that the loss of kairomonal activity of host trails is not due to microbially induced changes of the CHC pattern of a trail. Interestingly, the kairomonal activity of trails consisting of host larval CHC extracts was recoverable after two days by applying hexane to them. After hexane evaporation, the parasitoids followed the reactivated host trails as they followed freshly laid ones. Cryo-scanning electron microscopy showed that the trails gradually formed filament-shaped microstructures within two days. This self-assemblage of CHCs was reversible by hexane application. Our study suggests that the long-chain CHCs of a host trail slowly undergo solidification by a self-assembling process, which reduces the accessibility of CHCs to the parasitoid's receptors as such that the trail is no longer eliciting trail-following behavior.

Keywords Bethylidae · Insect cuticle · Perception · Trail-following behavior · Tenebrionidae · *Tribolium confusum*

Introduction

Successful foraging behavior of parasitic wasps depends on recognition of host-associated cues indicating the presence and location of a potential host. On a long range, highly volatile feeding- or oviposition-induced host plant odors, as well as volatile cues released by the host itself, may guide host searching parasitoids (Hilker and McNeil 2008; Steidle and Van Loon 2003; Vinson 1998). On a short range, chemicals of low volatility may become important for host location (Colazza et al. 2014; Vinson 1998).

Chemical trails left by host insects on the plant surface or feeding substrate were shown to consist of low volatile, long-chain cuticular hydrocarbons (CHCs) (Colazza et al. 2007; Fürstenau and Hilker 2017; Rostás and Wölfling 2009). Several parasitoid species are known to recognize and follow host trails (Colazza et al. 2009; Howard et al. 1998; Lo Giudice et al. 2011; Rostás and Wölfling 2009). These trails are used as kairomones for host location, for host recognition, and even for host gender discrimination (Borges et al. 2003; Gomes Lagôa et al. 2020; Howard and Flinn 1990; Salerno et al. 2009). In addition to trails deposited by hosts, parasitoids also react to trails released by predatory insects and by conspecifics. They respond to trails left by predators by avoidance behavior (Nakashima et al. 2004) and

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exploit trails left by conspecifics for mate finding (Bernal and Luck 2007; Kapranas et al. 2013).

CHCs of host insects chemically make up the dominant part of trails used by parasitoids (Blomquist and Bagnères 2010; Geiselhardt et al. 2009; Gomes Lagôa et al. 2020). Insects release their CHCs onto the substrate, which is in contact with their whole body or only their tarsi (e.g., Hasenfuss 1977; Geiselhardt et al. 2009, 2010; Gerhardt et al. 2016; Lo Giudice et al. 2011; Rostás and Wölfling 2009). In general, CHCs have several important functions in insects, ranging from protection against desiccation to the mediation of intra- and interspecific communication (e.g., Gibbs 1998; Howard and Blomquist 2005; Lockey 1988; Menzel et al. 2017; Otte et al. 2018). The multifunctionality of CHCs is based on the various types of CHCs, which can be saturated or unsaturated, linear or methyl-branched (Blomquist and Bagnères 2010; Blomquist and Ginzel 2021; Gibbs 2002). According to Menzel et al. (2019), the epicuticular layer of hydrocarbons forms a solid-liquid mixture over a broad range of temperatures due to the different melting temperatures of CHC types. Thus, the physical traits of CHCs vary with fluctuating environmental conditions to maintain the biological function of CHCs (Beament 1958; Menzel et al. 2017; Sprenger et al. 2018).

The persistence of kairomonal activity of host insect trails mediating the foraging behavior of parasitoids is limited in time, despite the low volatility of CHCs. Previous studies have shown that the kairomonal effect of host CHC trails on foraging larval parasitoids decreased significantly one day after trail deposition and lasted only for a maximum of three days (Fürstenau and Hilker 2017; Rostás and Wölfling 2009). The persistence of trails laid by social insects for recruitment of nestmates to resources is usually achieved by renewal of the trail as long as the resource is available. Thus, trails of social insects consisting of more volatile compounds than long-chain CHCs can persist for a long time but would decay within minutes if not consistently renewed (Jeanson et al. 2003; Morgan 2009; Robinson et al. 2008). However, trails left by non-social host insects of parasitoids are only renewed when the host is moving by chance on the same track where it has been before.

It is unknown so far which factors contribute to the low persistence of the kairomonal effect of host insect trails on foraging parasitoids. Microbes present on the substrate or released by host insects when depositing the trail might contribute to a change in the chemical profile of trails. Microbes living in symbiosis with insects are well known to shape the production of CHCs of their hosts (Engl and Kaltenpoth 2018; Sprenger and Menzel 2020). Entomopathogenic fungi (e.g., *Beauveria bassiana* ([Bals.-Criv.] Vuill., Cordycipitaceae) and *Metarhizium anisopliae* ([Meschn.] Sorokin, Clavicipitaceae)) are known to metabolize CHCs as a carbon source for their growth, thereby alternating the CHC profiles of infected host insects (Lecuona et al. 1991; Napolitano and Juárez 1997; Pedrini et al. 2013). However, no knowledge is available on whether microorganisms are involved in the decrease of kairomonal activity of host insect trails consisting of CHCs.

Here, we addressed this gap in knowledge by studying the persistence of the chemical composition of CHC trails released by *T. confusum* (Du Val 1863) (Coleoptera: Tenebrionidae), under sterile conditions (excluding microbial activity) and non-sterile ones. The CHC trails left by larvae of this beetle are followed by the larval ectoparasitoid *Holepyris sylvanidis* (Bréthes 1913) (Hymenoptera: Bethylidae), which attacks larvae of several stored-product beetles, including different species of the genus *Tribolium* (Amante et al. 2017, 2018; Evans 1969; Fürstenau and Hilker 2017). *Holepyris sylvanidis* discriminates between a host and a non-host species by a specific pattern of methyl-branched alkanes. The host-specific CHC pattern is common to the cuticle of different host beetle species (Awater-Salendo et al. 2020). Hexane extracts of host larvae deposited as trails elicit trail-following behavior of *H. sylvanidis*. The kairomonal effect of *T. confusum* larval trails on *H. sylvanidis* females was shown to last for at maximum two days, although these trails are exclusively composed of long-chain, low volatile, saturated CHCs (Fürstenau and Hilker 2017).

In detail, our study investigated the following questions by analyzing CHC trails of *T. confusum* larvae extracted with hexane: (1) Does the chemical composition of trails change over time past trail deposition when microbial degradation is excluded? (2) Does the chemical composition of trails from host larval extracts, which are not excluded from microbial degradation, change over time past trail deposition? Therefore, we analyzed the quantitative and qualitative CHC profiles of sterile-filtered and non-filtered CHC trails 0 h, 24 h, and 48 h after deposition by coupled gas chromatography-mass spectrometry (GC-MS). Furthermore, we asked (3) whether the kairomonal activity of 48 h-old, inactive trails from host larval extracts can be recovered by the application of hexane and (4) whether changes in physical structures of trails from host larval extracts 1 h and 48 h past deposition can be visualized by cryo-scanning electron microscopy (cryo-SEM).

Methods and Material

Insects

Individuals of *H. sylvanidis* and *T. confusum* were taken from a permanent rearing maintained at the Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection (Julius Kühn Institute, Berlin, Germany). The insects were reared on wheat grist according to a protocol

described by Fürstenau et al. (2016). For chemical analyses of host larval trails, we used 4th instar *T. confusum* larvae, which represent the preferred host stage of the parasitoid (Awater-Salendo et al. 2020). For the trail-following bioassays, we used unmated, one- to five-day-old *H. sylvanidis* females without previous oviposition experience.

Preparation of CHC Extracts from *T. confusum* Host Larvae

Since hexane extracts of *T. confusum* larvae elicit trail-following behavior in female *H. sylvanidis* when freshly applied onto a substrate but lose their kairomonal activity after two days (Fürstenau and Hilker 2017), we here studied whether the chemical composition of these trails changes over time past deposition. We used hexane extracts of 4th instar larvae (hereafter referred to as "larval trails") instead of naturally laid trails by larvae for our analyses because thus the quantities of compounds detected in the trails could be exactly determined and referred to larval individuals (larval equivalents = LE per volume hexane; see below).

The chemical composition of larval trails was analyzed 0 h, 24 h, and 48 h after trail deposition on glass Petri dishes (for details, see below). We analyzed freshly laid trails (0 h), i.e., the trails were extracted from the substrate immediately after deposition, and "aged" trails (24 h, 48 h), i.e., the trails were extracted from the substrate 24 h and 48 h after deposition. During these time intervals (24 h, 48 h), the trails were kept at room temperature and approximately 30.5 to 35.2% RH. In addition to the question of whether the chemical composition of trails changes over time past deposition, we also investigated whether the in- and exclusion of microbes affect the chemical trail composition. For GC-MS analyses, we, therefore, prepared (1) hexane extracts, which were sterile-filtered, and (2) non-sterile-filtered extracts. For trail-following bioassays with *H. sylvanidis* females and microscopic imaging of larval trails, we prepared non-sterile-filtered larval extracts (3).

For the preparation of stock solutions, the number of *T. confusum* larvae extracted varied according to their availability. However, we extracted a pool of larvae with always a defined number of individuals per microliter. This allowed us to calculate the exact number of LE per microliter finally deposited per trail. For all extracts (stock solutions), larvae were first killed by freezing them at −20 °C for up to 30 min and then thawed for ca. 2 min at ambient temperature. Thereafter, larvae were extracted by gentle shaking them for 10 min in *n*-hexane (analytical purification >98%, VWR, Radnor, USA). By this procedure, the layer of superficial CHCs is removed from larval integuments and dissolved in hexane (Fürstenau and Hilker 2017). The supernatant was further processed. The detailed protocols for the preparation of the above-mentioned three types of extracts are provided below. All samples prepared for GC-MS analysis, bioassays, or microscopic imaging were stored at −20 °C prior to usage.

(1) Sterile *T. confusum* hexane extracts for chemical analysis. The preparation of these extracts aimed to exclude any possible effects of microorganisms originating from the host (e.g., microbes on the cuticle of *T. confusum* larvae), the host habitat (here: wheat grist), or the environment (e.g., airborne microorganisms) on the CHC composition of host trails after different time intervals.

In total, five stock solutions were prepared. The supernatant was removed from the stock solution with a disposable syringe and loaded onto a sterile PVDF filter (0.22 µM, Carl Roth, Karlsruhe, Germany), which had been pre-conditioned by rinsing it with 10 ml *n*-hexane. Syringes were cleaned three times with *n*-hexane before being used. The PVDF filter loaded with larval extract was eluted with *n*-hexane. Sterile-filtered extracts were then concentrated to dryness under a gentle stream of nitrogen, dissolved in *n*-hexane, and frozen at −20 °C until further processing.

Under a clean bench, a trail of the sterile-filtered hexane extract was applied to the periphery of the bottom part of a glass Petri dish (diameter: 90 mm). The dish had previously been cleaned with demineralized water and 70% ethanol solution (analytical purification >96.0%, Berkel AHK, Ludwigshafen, Germany) and subsequently sterilized at 175 °C for 3.5 h. After trail application, the solvent was allowed to evaporate for 1 min. We prepared five replicates (Petri dishes, $N = 5$) for each investigated time interval past trail deposition. For the 0 h interval, trails were re-extracted in *n*-hexane immediately after deposition, transferred to a 2 ml vial, evaporated to dryness under a gentle stream of nitrogen, and then dissolved in a distinct volume of *n*-hexane with 1-eicosene as an internal standard (IS, Sigma-Aldrich, Taufkirchen, Germany). For trails to be investigated 24 h and 48 h after trail deposition, the dishes were sealed with Parafilm® and kept in a closed sterile box outside the sterile bench. After 24 h and 48 h storage, trails were removed from the dishes as described for the immediately analyzed trails.

(2) Non-sterile *T. confusum* hexane extracts for chemical analysis. To imply possible effects of host-associated or environmentally present microorganisms on the CHC composition of host trails at different time intervals past trail deposition, larval hexane extracts were prepared under non-sterile conditions.

In total, we prepared six stock solutions. In contrast to the sterile-filtered extracts, here the supernatants were not filtered but directly concentrated to dryness under a gentle

stream of nitrogen, dissolved in *n*-hexane, and frozen at −20 °C for further analysis.

As described for the sterile extracts, trails were deposited on eighteen Petri dishes ($N = 6$ per time interval) which had been cleaned but not sterilized by heat prior to application. After solvent evaporation, Petri dishes were kept open under a laboratory hood for 0 h, 24 h, or 48 h (non-sterile conditions). After the respective time intervals, larval trails were re-extracted in *n*-hexane and quickly transferred to a vial. These extracts were then concentrated under a gentle stream of nitrogen to dryness, and re-dissolved in a distinct volume of *n*-hexane containing again 1-eicosene as internal standard.

(3) Non-sterile *T. confusum* hexane extracts for bioassays and microscopic imaging. We studied the parasitoid's behavioral responses to larval trails in dependence (a) of the time intervals past deposition ("untreated trails") and (b) of re-dissolving "aged" trails by *n*-hexane ("re-dissolved trails") (see below for bioassay method). For each type of trail, we prepared seven non-sterile-filtered stock solutions ($N = 7$).

For cryo-SEM (see below), we took the same samples from the stock solutions of larval extracts.

GC-MS Analysis of Sterile- and Non-sterile-Filtered *T. confusum* Host Larval Trails

Samples were analyzed on a 6890 N GC coupled to a 5975 B VL MS quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany). As carrier gas, we used helium with a flow rate of 1.1 ml min^{-1} and a fused silica column (HP-5MS capillary column, 30 m × 0.25 mm × 0.5 µm, Agilent Technology, Waldbronn, Germany) as a stationary phase. One µl of each sample was injected at 250 °C in splitless mode. The oven temperature program started at 40 °C for 4 min, then raised by 10 °C min^{-1} up to 300 °C, which was held for 10°min. After a solvent delay of 5 min, the detector scanned 4.45 times s^{-1} for fragments in a range from 35 to 500 *m/z* (electron impact [EI] ionization = 70°eV).

Linear alkanes were identified by comparing their retention indices (RIs) and mass spectra with those of an authentic *n*-alkane standard (*n*-C7 - *n*-C40, Sigma Aldrich, Taufkirchen, Germany). Since no reference standards of methyl-branched alkanes were available to us, we tentatively identified the detected ones based on their characteristic mass spectrometric fragmentation pattern and RIs calculated according to Van den Dool and Kratz (1963). Additionally, RIs and fragmentation pattern of these substances were compared to those published by Fürstenau and Hilker (2017). Individual compounds (linear and methyl-branched alkanes) were quantified relative to the peak area of the IS (1-eicosene).

Trail-Following Bioassays

To analyze the trail-following responses of *H. sylvanidis* females to *T. confusum* larval trails, we used a "walking arena", i.e. the lower part of a glass Petri dish (diameter = 190 mm). We modified the experimental set-up described by Fürstenau and Hilker (2017) as follows. The rim of each Petri dish was coated with an aqueous solution of Teflon (Sigma Aldrich, Taufkirchen, Germany) to prevent parasitoids from climbing up during the bioassays. Below the bottom of the dish, we attached a drawing of two circles (diameter: 80 mm each) as a template for the trails (one test and one control trail) to be laid on the glass side. A test trail consisted of a hexane extract of *T. confusum* larvae, while a control trail consisted of only hexane. The two circles were 30 mm apart from each other, and they were about 3 mm wide on the periphery. Additionally, each circle was divided into eight equally sized sections (length: 31 mm each). A strip of light-emitting diodes ($\lambda = 625$ nm, Barthelme GmbH & Co, Nuremberg, Germany) was located 300 mm above the arena for consistent illumination.

We tested the trail-following responses of *H. sylvanidis* females to two different types of larval trails: (1) larval trails at different time intervals after deposition (0 h, 24 h, and 48 h); ("untreated trails"); and (2) larval trails at different time intervals after deposition (0 h and 48 h); these trails had been treated with *n*-hexane just prior to exposure to the parasitoids ("re-dissolved trails"). All bioassays were performed at room temperature and approximately 35 to 40% RH on three consecutive days.

When testing untreated trails, we deposited 25 µl (corresponding to 5 LE) of a stock solution on a test circle in the Petri dish. We knew from our previous study that *H. sylvanidis* shows trail-following behavior already to very low-concentrated larval extracts (Fürstenau and Hilker 2017). The control trail consisted of 25 µl *n*-hexane. Each stock solution was used once for each time interval and type of trail ($N = 7$). Host larval trails tested at later time intervals after deposition were kept under a laboratory hood in open Petri dishes for 24 h or 48 h prior to the beginning of the respective bioassays. A bioassay started after releasing one *H. sylvanidis* female at a randomly selected position between the circular control and test trail and lasted 300 s.

Since the results of the bioassay with "untreated trails" revealed that differences in the parasitoid's response were the greatest between 0 h and 48 h after trail deposition, we decided to use only these two time intervals in the bioassay with "re-dissolved trails".

When testing the "re-dissolved trails", test and control trails were first applied to the dishes as described for

"untreated trails". Thereafter, just prior to testing, 25 µl additional *n*-hexane was applied on the circular test and the control trail. The hexane was allowed to evaporate for 1 min. The bioassay started then by releasing one *H. sylvanidis* female into the walking arena as described above.

When parasitoid females exhibited trail-following behavior, they intensely antennated host larval trails and followed them in a zigzag line (Howard and Flinn 1990). *Holepyris sylvanidis* females also tended to reverse the walking direction or to stop following a trail to explore the local area before returning to the trail. Therefore, we evaluated the behavioral response of *H. sylvanidis* to each treatment based on two parameters: (1) the distance covered by a parasitoid was quantified by the number of sections, which a parasitoid fully walked on test and control circles; (2) the total time spent by a parasitoid on each trail was recorded using the computer software "The Observer 3.0" (Noldus Information Technology, Wageningen, The Netherlands). After each run, we replaced the tested individual and changed the position of control and test circle to avoid any biased results due to possible side preferences. After having tested four parasitoids, the bioassay arena was replaced by a new one. When freshly applied trails (= 0 h) were tested, we reused the test arena after cleaning it with demineralized water and a 70%-ethanol solution. Parasitoids, which rested more than 50% of the observation time, were excluded from the statistical analysis and replaced by a new one. This was the case in less than 4% (5 occasions) of all experiments. In total, we tested each type of trail at each time interval past deposition with 28 female *H. sylvanidis* ($N = 28$).

Cryo-SEM

To study possible changes in physical structure of CHC-consisting trails at different time intervals past trail deposition, the cryo-SEM SUPRA 40VP-31-79 (Carl Zeiss SMT Ltd., Oberkochen, Germany) equipped with an EMITECH K250X cryo-preparation unit (Quorum Technologies Ltd., Ashford, Kent, UK) was used.

Hexane extracts of host larvae were applied onto either a polar substrate or a non-polar one. We used the polar substrate because (i) we here always applied host larval trails on glass (polar) when studying the parasitoids' behavioral response to them and (ii) *T. confusum* might encounter polar substrates in nature (e.g., fine flour with its carbohydrates). We used also an apolar substrate because these substrates might also be present in the natural habitat of *T. confusum* larvae (e.g., waxy seed coats). The polar substrate consisted of 5×5 mm pieces of ultra-flat silicon wafer thermically covered with a 200 nm thick, polished SiO_2 film (surface roughness: 2–3 Å) (Plano GmbH, Wetzlar, Germany). The substrate had been cleaned prior to use by successive immersions in Piranha solution (mixture of sulphuric acid H_2SO_4 and hydrogen peroxide H_2O_2, 3:1), rinsed with distilled water, and dried by compressed air. To obtain apolar substrates, which are known to promote wax crystallization (e.g., Niemietz et al. 2009), we silanized the polar substrate with 1H,1H,2H,2H perfluorodecyltrichlorosilane ($C_{10}H_4Cl_3F_{17}Si$, 97%, SIH5841.0, ABCR GmbH & Co. KG, Karlsruhe, Germany).

The CHC larval extract was slowly applied in 5 µl steps onto the substrate. In total, 25 µl could be placed onto the small piece of substrate. The extracts were kept for 1 h or 48 h on the substrates at ambient temperature and $25.7 \pm 2.79\%$ RH until further processing ("untreated trails"). In addition, we applied a droplet of hexane to samples with larval extracts, which had been on the substrate for 1 h or 48 h ("re-dissolved trails").

For cryo-SEM analysis, the substrates were mounted on metal stubs using polyvinyl alcohol (Tissue-Tek, OCT, Sakura Finetek Europe BV, Alphen aan den Rijn, the Netherlands). The solvent was evaporated for at least 1 h to avoid interferences inside the cryo-SEM. Subsequently, the samples were shock-frozen in liquid nitrogen in the slushing chamber, transferred to the cryo-preparation chamber at −140 °C, sublimed for 15 min at −70 °C, sputter-coated with platinum (layer thickness ca. 10 nm), transferred to the SEM, and then examined in a frozen state at 5 kV accelerating voltage and − 100 °C temperature. Cryo-SEM micrographs were taken using the software Smart SEM 05.03.05 (Carl Zeiss SMT Ltd., Oberkochen, Germany).

Statistical Analysis

We conducted all statistical analysis using R, version 4.0.2 (R Core Team 2020), except for the analysis of similarity percentages (SIMPER), which was performed in "PAST", version 3.26 (Hammer et al. 2001).

For statistical comparison of the quantitative chemical pattern of sterile- or non-sterile-filtered trails, we selected the analyzed compounds by the following criteria: (i) presence in more than 50% of all samples taken per time interval past deposition; (ii) peak area larger than 0.01% of the total peak area. If a compound was meeting criterion (i), but not criterion (ii) (i.e., the compound was below the detection limit), the "in some samples, missing value" was handled as follows. We used the "*rnorm()*"-function in R to generate a random peak for each missing value. We selected the smallest peak area, which the missing value had in other samples of the same time interval and trail type (sterile- or non-sterile-filtered), as mean and calculated the standard deviation based on the three smallest peak areas. To avoid any bias in the subsequent statistical analyses, nine pseudo-peaks were generated in the samples of sterile-filtered larval trails, while one pseudo-peak was used for non-sterile-filtered larval trails. We normalized the peak areas of selected compounds

by referring the quantity (peak area) of each compound to the IS and then to one larval equivalent (LE).

To assess whether the chemical composition of sterile-filtered larval CHC trails varied quantitatively over time, quantities of each selected compound were compared by a one-way ANOVA when data were normally distributed. If the *Shapiro-Wilk* test of normality revealed that data of some compounds were not normally distributed at all time intervals, the *Kruskal-Wallis* test was computed instead, followed by *Wilcoxon's* rank-sum test with *Bonferroni-Holm* correction. Furthermore, the dissimilarity in the chemical composition of these larval trails was tested by running a one-way analysis of similarity (ANOSIM). For this purpose, we calculated the relative amounts of detected compounds in one LE per time interval and summed them up to 100%. Based on *Bray-Curtis* dissimilarity, an ANOSIM was performed with 99,999 random permutations using the package "vegan" (version 2.5–6, Oksanen et al. 2019) in R. The dissimilarity of groups is stated by the *R*-value; groups with an *R*-value close to 0 are highly similar, while groups with an *R*-value close to 1 can be clearly discriminated (Clarke 1993). For visualization of results obtained by the ANOSIM, a non-metric multidimensional scaling (NMDS) was calculated based on the *Bray-Curtis* dissimilarity. To evaluate how well the algorithm of NMDS fits in the used data set, we calculated the associated stress value. According to Dexter et al. (2018), a stress value <0.1 indicates that the applied NMDS is a good representative. A SIMPER was implemented to identify compounds that contributed the most to the dissimilarity. Likewise, the statistical analysis of trails from non-sterile-filtered larval extracts was performed.

For each time interval, the quantitative chemical compositions of sterile- and non-sterile-filtered larval CHC trails were statistically analyzed by using a *Student's t*- test for independent data. When the variance was unequal in both data sets, a *Welch's t*-test was applied instead. If data of one CHC trail type were not normally distributed according to the *Shapiro-Wilk* test of normality, we used *Wilcoxon's* rank-sum test for independent data.

The behavioral responses of *H. sylvanidis* to host larval trails were statistically analyzed (a) by comparing the walking distances and residence times on test circles with the CHC trail and on control circles with the solvent. These parameters were statistically compared by a *Student's t*- test for paired samples. If data sets were not normally distributed according to the *Shapiro-Wilk* test, we performed a *Wilcoxon's* signed-rank test for paired data. Furthermore, we compared (b) whether walking distances and residence times on test circles differed when trails were offered 0 h, 24 h, or 48 h after deposition. Therefore, we determined the difference (Δ) in walking distance and residence time spent on the test and the control circle for each time interval. When analyzing the responses to "untreated test and control trails", the differences in residence times and walking distances in bioassays with trails tested 0 h, 24 h, or 48 h after deposition were not normally distributed according to the *Shapiro-Wilk* test. Therefore, we performed a *Kruskal-Wallis* test for each parameter (walking distance, residence time) followed by a pairwise *Wilcoxon's* rank-sum test with *Bonferroni-Holm* correction. When comparing the responses to "re-dissolved trails" on test circles 0 h and 48 h after deposition, we applied a *Student's t*- test for each parameter (walking distance, residence time) if the data were normally distributed. If normal distribution of data was absent, a *Wilcoxon's* rank-sum test was performed.

Results

No Changes in the Chemical Composition of *T. confusum* Host Larval Trails in the Course of Time

We addressed the question of whether host larval trails, which had been excluded from microbial degradation, changed their chemical composition in the course of time (0 h, 24 h, and 48 h) past deposition. Our chemical analysis revealed the same 20 compounds in all investigated sterile-filtered trails, regardless of the time past trail deposition (Table 1).

The detected trail compounds included six *n*-alkanes, twelve monomethyl-branched alkanes, and two dimethyl-branched alkanes with a chain length from C25 to C31. In all CHC profiles of larval trails and at all time intervals past trail deposition *n*-alkanes were the dominating substance group accounting for 80%, while methyl-branched alkanes accounted for about 20% (Table 1). The quantities of individual CHCs did not differ significantly between freshly laid trails (0 h), 24 h- and 48 h-old ones. Additionally, the ANOSIM confirmed that the CHC compositions of larval trails were highly similar at all time intervals past trail deposition ($R = -0.141$, $P = 0.941$). CHC profiles of these trails clustered closely in a NMDS plot calculated on the relative proportions of single compounds within one LE of *T. confusum* (Fig. 1a). According to the SIMPER analysis, *n*-C25, *n*-C27, and *n*-C29 (entries 1, 8, and 18, Table 1) contributed the most to the moderate dissimilarity (59%, Table S1). Hence, at sterile conditions, CHC profiles of freshly laid host larval trails and those, which were 24 h or 48 h old, did not significantly differ. The CHC profiles did not change within two days.

We further addressed the question of whether CHC profiles of host larval trails kept under non-sterile conditions change over time. The detected compounds of non-sterile trails after the different time intervals past deposition (Fig. 2) included linear alkanes, which accounted for almost 70% in CHC profiles of these trails, and methyl-branched alkanes,

Table 1 Cuticular hydrocarbons identified from sterile-filtered hexane extracts Of *Tribolium confusum* larvae 0 H, 24 H OR 48 H after trail (extract) deposition. Mean amounts (NG ± SE LE^{-1}) And Relative Quantities (% PER LE) are given

					Hours after trail deposition[f]						
					0		24		48		
No.[a]	Compound[b]	ID[c]	RI_{cal}[d]	RI_{lit}[e]	Mean ± SE (ng)[g]	(%)[g]	Mean ± SE (ng)[g]	(%)[g]	Mean ± SE (ng)[g]	(%)[g]	P^h
1	*n*-C25	I	2498	2500	20.52 ± 3.04	17.22	17.99 ± 3.53	17.14	21.83 ± 2.40	17.63	ns
2	11–/13-MeC25	II	2533	2534	0.13 ± 0.03	0.11	0.13 ± 0.03	0.12	0.15 ± 0.03	0.12	ns
3	5-MeC25	III	2550	2550	0.08 ± 0.02	0.07	0.08 ± 0.02	0.07	0.10 ± 0.01	0.08	ns
4	3-MeC25	IV	2573	2571	0.40 ± 0.09	0.33	0.36 ± 0.10	0.33	0.40 ± 0.08	0.32	ns
5	*n*-C26	V	2598	2599	3.10 ± 0.43	2.58	2.61 ± 0.54	2.45	3.07 ± 0.34	2.48	ns
6	10–/11–/12–/13-MeC26	VI	2633	2632	0.53 ± 0.04	0.46	0.46 ± 0.11	0.43	0.51 ± 0.09	0.41	ns
7	4-MeC26	VII	2656	2656	0.27 ± 0.03	0.23	0.21 ± 0.06	0.21	0.22 ± 0.05	0.18	ns
8	*n*-C27	VIII	2700	2700	53.27 ± 5.70	45.07	46.66 ± 7.15	45.49	57.52 ± 5.41	46.65	ns
9	11–/13-MeC27	IX	2730	2731	8.10 ± 0.75	6.95	7.25 ± 1.51	6.91	8.61 ± 1.12	6.91	ns
10	5-MeC27	X	2747	2750	3.81 ± 0.32	3.28	3.35 ± 0.65	3.25	3.84 ± 0.45	3.11	ns
11	3-MeC27	XI	2771	2773	3.50 ± 0.39	2.97	3.07 ± 0.59	2.94	3.58 ± 0.38	2.89	ns
12	5,X-DiMeC27	XII	2778	2781	1.95 ± 0.15	1.69	1.71 ± 0.37	1.64	1.86 ± 0.27	1.50	ns
13	*n*-C28	XIII	2797	2799	4.86 ± 0.53	4.11	4.23 ± 0.71	4.11	4.97 ± 0.50	4.03	ns
14	3,X-DiMeC27	XIV	2803	2807	2.64 ± 0.18	2.28	2.15 ± 0.49	2.04	2.42 ± 0.31	1.97	ns
15	12–/13–/14-MeC28	XV	2828	2831	0.93 ± 0.07	0.81	1.07 ± 0.32	1.02	0.70 ± 0.14	0.56	ns
16	4-MeC28	XVI	2854	2856	0.54 ± 0.03	0.47	0.47 ± 0.13	0.46	0.36 ± 0.07	0.30	ns
17	*n*-C29	XVIII	2897	2904	11.77 ± 1.28	9.99	10.42 ± 1.86	10.04	11.96 ± 1.20	9.71	ns
18	11–/13-MeC29	XIX	2928	2931	1.25 ± 0.12	1.07	1.15 ± 0.29	1.06	0.93 ± 0.09	0.78	ns
19	3-MeC29	XXI	2970	2978	0.23 ± 0.05	0.20	0.27 ± 0.11	0.22	0.31 ± 0.06	0.27	ns
20	*n*-C31	XXV	3096	3100	0.13 ± 0.01	0.12	0.08 ± 0.04	0.07	0.10 ± 0.01	0.08	ns

[a]Number of peaks identified in the total ion chromatogram

[b]*n*-alkanes were identified by comparing RIs and mass spectra with authentic standards. Methyl-branched alkanes were tentatively identified by the diagnostic ions, which resulted from favored fragmentation at branched points (see by Fürstenau and Hilker 2017, and by comparing RIs with data from literature

[c]Identity of CHCs used for comparison of sterile- and non-sterile-filtered larval host trails

[d]RI_{cal} = Retention index calculated on a HP-5 ms capillary column (30 m × 0.25 mm × 0.5 µm)

[e]RI_{lit} = Retention index as reported for compounds analyzed on HP-5 ms or similar columns in the database (http://www.pherobase.com/) and by Fürstenau and Hilker (2017)

[f]For the preparation of host larval trails, see experimental part

[g]For each time interval, five replicates were used ($N = 5$)

[h]For each compound, a *p* value denotes a significant quantitative difference between sterile-filtered larval CHC trails of *T. confusum* 0 h, 24 h, and 48 h after trail deposition (one-way ANOVA or *Kruskal-Wallis* test, ns = not significant)

which made up about 30% of the detected CHCs (Table 2). No significant differences were measured between the relative quantity of individual compounds present in freshly laid trails (0 h) and in "aged trails" 24 h and 48 h after deposition. As a result, the CHC profiles clustered closely in a NMDS plot (Fig. 1b). This finding indicated a strong similarity of the chemical composition of non-sterile-filtered larval trails analyzed after different times past deposition. This similarity was confirmed by the results of the ANOSIM ($R = -0.144$, $P = 0.953$). The SIMPER analysis revealed that *n*-C25, *n*-C27, 11–/13-MeC27, and 5-MeC27 (entries 1, 8, 9, and 10) contributed the most to the moderate dissimilarity (57%, Table S2). Thus, at non-sterile conditions, no significant differences were detected in the qualitative and quantitative CHC pattern of 0 h-, 24 h- and 48 h-old trails of *T. confusum* larvae. The long-chain CHC profiles were stable over a period of two days.

When comparing the chemical composition of sterile- and non-sterile-filtered trails for each time interval past trail deposition, we found that sterile-filtered trails were missing five compounds, which were detected in the non-sterile-filtered trails (ID XVII, XX, XXII, XXIII, and XXIV in Table 2). When statistically comparing the quantities of the compounds present in both CHC trail types, we found that at all time intervals the quantity of individual CHCs was significantly higher in non-sterile-filtered trails than in sterile

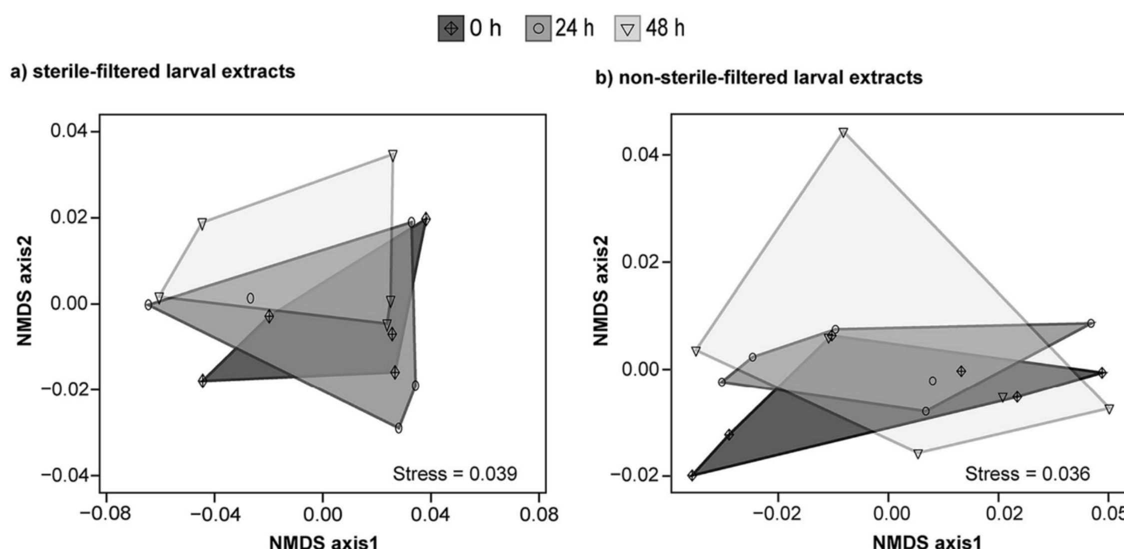

Fig. 1 Non-metric multidimensional scaling (NMDS) visualization of the CHC composition of trails consisting of two differently treated *Tribolium confusum* larval extracts 0 h, 24 h, and 48 h after trail deposition: **a)** sterile-filtered hexane extracts, which were applied and kept under sterile conditions ($N = 5$) and **b)** non-sterile-filtered hexane extracts, which were applied and kept under non-sterile conditions ($N = 6$)

ones (for *p*-values see Table S3). Only the quantities of *n*-C25 and *n*-C27 (ID I and VIII in Table S3) did not significantly differ between both CHC trail types at the respective time intervals.

Change of Kairomonal Activity of Host Trails in the Course of Time

Our bioassays confirmed previous results of Fürstenau and Hilker (2017) and show a clear kairomonal activity of freshly laid CHC trails, but a decrease of the kairomonal effect within two days after trail deposition. Parasitoid females covered a significantly greater distance on freshly laid CHC trails than on “aged” 24 h- or 48 h-old ones (Fig. 3a). When comparing the walking distance on CHC-consisting test trails and (hexane) control trails, significantly greater distances were covered on CHC trails 0 h and 24 h after trail deposition, while this preference was lost 48 h after trail deposition (Table S4). When comparing the residence time on test and control trails, the residence time on test trails was always significantly higher than on control trails (Table S4). However, the time spent by the parasitoids on test trails decreased considerably with the “age” of a trail. Parasitoids spent significantly more time on freshly laid CHC trails than on 24 h- or 48 h-old ones (Fig. 3b).

We further studied whether the kairomonal effect of 48 h-old larval trails could be reactivated by applying hexane to them. Parasitoids covered a similar walking distance and spent similar time on 48 h-old CHC trails with hexane reapplication as on freshly laid (0 h), untreated CHC trails (Fig. 4a, b). When comparing the parasitoid’s response to 48 h-old CHC trails with hexane reapplication to the hexane control trails, the results show that they covered a greater distance on re-dissolved 48 h-old test trails than on the controls and spent significantly more time on these trails (Table S4). Hence, an application of hexane fully restored the kairomonal activity of 48 h-old CHC trails.

Change of Trail Structures in the Course of Time

We investigated by cryo-SEM whether trails obtained from hexane extracts of *T. confusum* larvae show changes in physical structures over time past trail deposition.

Both on a polar and non-polar substrate, untreated trails changed their microscopically visible structures within 48 h (Fig. 5A1-A3, B1–3). One hour after application of the CHC trail onto the substrate, fluid patches or droplets were visible (Fig. 5A2, B2). On the polar substrate, the larval trail extract formed a ring-shaped pattern, which occurred 1 h after deposition and evaporation of hexane; such a formation has also been described as “coffee-ring effect” (Deegan et al. 1997, 2000). After 48 h, solidified filamentous structures were visible (Fig. 5A3). The filamentous structures were visible as clearly outlined,

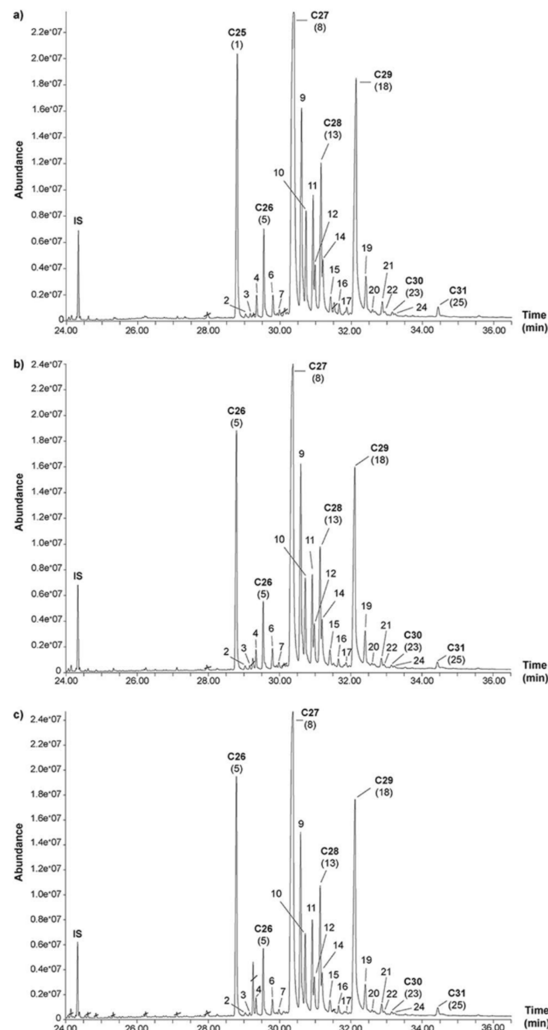

Fig. 2 Partial total ion chromatograms (TIC) of trails consisting of non-sterile-filtered hexane extracts of *Tribolium confusum* larvae; trails (hexane extracts) were extracted from a substrate and analyzed **a**) 0 h, **b**) 24 h, and **c**) 48 h after deposition. Numbers above peaks refer to the identified compounds listed in Table 2. The *n*-alkanes (*n*-C25 – *n*-C31) detected in the larval trails are highlighted in bold. Crossed-out compounds are contaminations

elongated threads on the polar substrate, while they were embedded in a solidified, amorphous layer with a gibbous surface on the apolar substrate (Fig. 5B3).

When applying hexane to a 48 h-old CHC trail, again fluid-like structures of these re-dissolved trails were visible, i.e. a fluid layer with granules on the polar substrate (Fig. 5A4) and droplets on the apolar substrate (Fig. 5B4).

Discussion

Our present study addressed the question of whether the quick loss of kairomonal activity (within two days) of host larval trails used by a foraging larval parasitoid is due to chemical changes which might be caused by microbial activity. Our GC-MS analyses revealed that the chemical pattern of trails from *T. confusum* larval extracts (hereafter referred to larval trails) does not change over a period of 48 h after trail deposition, regardless of whether the possible microbial activity was excluded or not. Thus, no hints were detected (i) on chemical degradation of host CHC trails, (ii) on microbial contribution to a change in the chemical composition of the trails, and (iii) on the quantitative loss of CHCs due to e.g., evaporation. Our bioassays showed that the kairomonal activity of non-sterile CHC trails was restored by adding hexane as solvent to an inactive 48 h-old trail. These reactivated larval trails induced a comparable trail-following behavior in *H. sylvanidis* as we could observe with freshly laid trails. Cryo-SEM analysis showed that the CHC trails formed filamentous structures 48 h after the trail deposition, which were re-dissolved by hexane and then forming fluidic layers or droplets. Our results suggest that the quick loss of kairomonal activity of host larval trails after a short time is neither due to microbial degradation nor to a chemical change in the CHC profile, but due to a shift in the physical state and molecular packing of the CHC blend. The observed gradual assembling of host larval CHCs in filamentous structures after trail deposition might render the CHCs less accessible to the olfactory receptors by which insects perceive CHCs (Ozaki and Wada-Katsumata 2010). Such a reduced perceptibility is expected to reduce the kairomonal activity of the trails.

The physical states of individual, long-chained CHCs range from liquid to solid at ambient temperature. The CHC profile of *T. confusum* larvae is dominated by *linear* alkanes with a chain length from C25 to C31. These CHCs are known to form solid structures at ambient temperature and start melting at temperatures above 50 °C (Gibbs and Pomonis 1995; Maroncelli et al. 1982). Some long-chain *monomethyl-branched alkanes* can also solidify at room temperature range (Brooks et al. 2015), but the molecular packing and thus the melting behavior of this CHC type depends particularly on the position of the methyl group (Gibbs 1998). For example, 3-MeC25 becomes liquid at 40 °C, whereas 11-MeC25 does at approx. 20 °C. *Dimethyl-branched alkanes* show the lowest melting temperatures among all CHC types present on the cuticle of *T. confusum* larvae and are most likely liquid at room temperature (Gibbs 1998; Gibbs and Pomonis 1995).

The *T. confusum* larval extracts deposited as trails consisted of a blend of different CHC types with different

Table 2 Cuticular hydrocarbons identified from non-sterile-filtered hexane extracts of *Tribolium confusum* larvae 0 h, 24 h or 48 h after trail (extract) deposition. Mean amounts (ng ± SE LE−1) and relative quantities (% per LE) are given

					Hours after trail deposition[f]						
					0		24		48		
No.[a]	Compound[b]	ID[c]	RI_{cal}[d]	RI_{lit}[e]	Mean ± SE (ng)	(%)	Mean ± SE (ng)	(%)	Mean ± SE (ng)	(%)	*P*[g]
1	*n*-C25	I	2503	2500	20.12 ± 0.67	11.26	19.47 ± 0.77	11.41	19.10 ± 0.49	11.25	ns
2	11−/13-MeC25	II	2534	2534	0.56 ± 0.06	0.31	0.46 ± 0.03	0.27	0.63 ± 0.10	0.37	ns
3	5-MeC25	III	2550	2550	0.36 ± 0.04	0.20	0.31 ± 0.02	0.18	0.38 ± 0.06	0.22	ns
4	3-MeC25	IV	2573	2571	1.31 ± 0.12	0.73	1.22 ± 0.06	0.71	1.28 ± 0.07	0.75	ns
5	*n*-C26	V	2599	2599	5.19 ± 0.23	2.90	5.02 ± 0.15	2.95	5.12 ± 0.15	3.01	ns
6	10−/11−/12−/13-MeC26	VI	2633	2632	1.44 ± 0.16	0.79	1.37 ± 0.09	0.80	1.50 ± 0.15	0.88	ns
7	4-MeC26	VII	2658	2656	0.79 ± 0.08	0.44	0.66 ± 0.05	0.39	0.82 ± 0.10	0.48	ns
8	*n*-C27	VIII	2708	2700	56.50 ± 2.77	31.50	53.37 ± 1.68	31.28	52.23 ± 0.78	30.75	ns
9	11−/13-MeC27	IX	2735	2731	15.75 ± 1.56	8.68	15.13 ± 0.90	8.86	14.72 ± 0.95	8.62	ns
10	5-MeC27	X	2750	2750	7.61 ± 0.65	4.20	7.24 ± 0.34	4.24	8.30 ± 1.19	4.85	ns
11	3-MeC27	XI	2774	2773	7.30 ± 0.30	4.07	7.17 ± 0.10	4.21	7.02 ± 0.16	4.13	ns
12	5,X-DiMeC27	XII	2781	2781	3.79 ± 0.35	2.09	3.47 ± 0.19	2.03	3.36 ± 0.18	1.97	ns
13	*n*-C28	XIII	2801	2799	10.45 ± 0.41	5.84	10.28 ± 0.18	6.03	10.18 ± 0.21	5.99	ns
14	3,X-DiMeC28	XIV	2806	2807	5.10 ± 0.35	2.83	4.82 ± 0.16	2.82	4.80 ± 0.23	2.82	ns
15	12−/13−/14-MeC28	XV	2830	2831	1.98 ± 0.19	1.09	1.86 ± 0.12	1.09	1.87 ± 0.18	1.09	ns
16	4-MeC28	XVI	2856	2856	1.50 ± 0.12	0.83	1.29 ± 0.05	0.76	1.35 ± 0.11	0.79	ns
17	3-MeC28	XVII	2873	2872	0.71 ± 0.16	0.39	0.44 ± 0.02	0.26	0.57 ± 0.08	0.33	ns
18	*n*-C29	XVIII	2904	2904	29.10 ± 1.57	16.18	28.06 ± 0.52	16.46	27.68 ± 0.33	16.28	ns
19	11−/13-MeC29	XIX	2930	2931	4.51 ± 0.43	2.49	4.06 ± 0.32	2.38	4.10 ± 0.39	2.40	ns
20	5-MeC29	XX	2948	2948	0.91 ± 0.11	0.50	0.70 ± 0.02	0.41	0.71 ± 0.05	0.41	ns
21	3-MeC29	XXI	2972	2978	1.40 ± 0.10	0.78	1.29 ± 0.05	0.76	1.34 ± 0.09	0.78	ns
22	5,X-DiMeC29	XXII	2979	2980	0.83 ± 0.05	0.46	0.65 ± 0.03	0.38	0.70 ± 0.06	0.41	ns
23	*n*-C30	XXIII	2999	3000	0.64 ± 0.03	0.35	0.54 ± 0.03	0.32	0.58 ± 0.05	0.34	ns
24	3,X-DiMeC29	XXIV	3004	3005	0.68 ± 0.07	0.38	0.55 ± 0.04	0.32	0.61 ± 0.12	0.36	ns
25	*n*-C31	XXV	3098	3100	1.29 ± 0.04	0.73	1.15 ± 0.10	0.68	1.19 ± 0.05	0.70	ns

[a]Peak numbers referring to Fig. 2

[b]*n*-alkanes were identified by comparing RIs and mass spectra with authentic standards. Methyl alkanes were tentatively identified by the diagnostic ions, which resulted from favored fragmentation at branched points (see by Fürstenau and Hilker 2017), and by comparing RIs with data from literature

[c]Identity of CHCs used for comparison of sterile and non-sterile-filtered larval host trails

[d]RI_{cal} = Retention index calculated on a HP-5 ms capillary column (30 m × 0.25 mm × 0.5 µm)

[e]RI_{lit} = Retention index as reported for compounds analyzed on HP-5 ms or similar columns in the database (http://www.pherobase.com/) and by Fürstenau and Hilker (2017)

[f]For the preparation of host larval trails, see experimental part

[g]For each time interval, six replicates were used ($N = 6$)

[h]For each compound, the p value denotes a significant quantitative difference between non-sterile-filtered larval CHC trails of *T. confusum* 0 h, 24 h, and 48 h after trail deposition (one-way ANOVA or *Kruskal-Wallis* test, ns = not significant)

physical states at ambient temperature. How might the physical state of this blend change within 48 h and lose its kairomonal activity?

We suggest the following scenario: After evaporation of the solvent hexane, the CHCs extracted from *T. confusum* host larvae began to rearrange themselves due to intermolecular interactions (e.g., van der Waals forces). These self-assembling processes might result in a more ordered state, and thus a change from liquid to solid occurred. The gradual decrease of the kairomonal activity of the larval trails suggests that the CHCs first formed a solid-liquid matrix in which some components had already solidified, whereas others were still present as liquids and therefore perceivable for *H. sylvanidis*. In the course of time, the CHC solidification might have gradually proceeded, thus rendering those CHCs informative to the parasitoid in their liquid phase no longer

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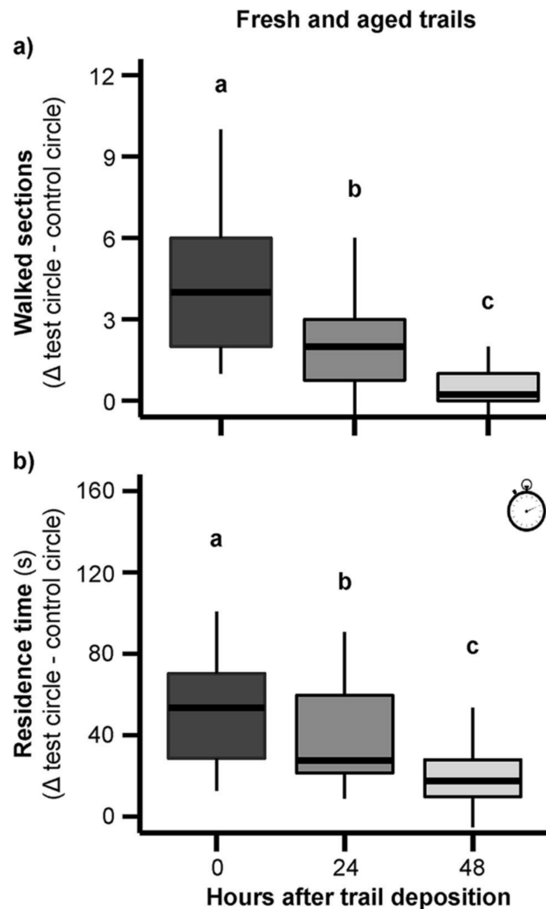

Fig. 3 Behavioral response of female *Holepyris sylvanidis* to trails consisting of non-sterile-filtered larval extracts of *Tribolium confusum* (5 LE of *T. confusum* 4th instar larvae per trail). Circular trails were deposited 0 h, 24 h, or 48 h before being offered to the parasitoids (N =28 per time interval). Mean differences (Δ) of **a)** walked sections and **b)** residence time (indicated by the clock inset) on test and control circle at different time intervals are displayed. Test circle: hexane-extracted larval host trails. Control circle: hexane only. The parasitoid's trail-following activity at different time intervals was analyzed by a *Kruskal-Wallis* test followed by pairwise *Wilcoxon's* rank-sum test with *Bonferroni-Holm* correction. Different letters indicate significant differences at $P < 0.05$

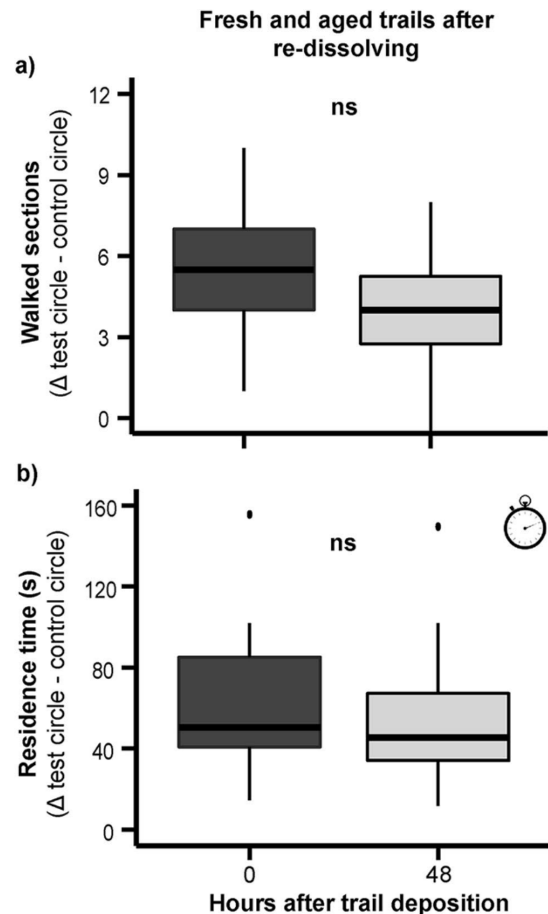

Fig. 4 Behavioral response of female *Holepyris sylvanidis* to hexane-redissolved trails from non-sterile-filtered larval extracts of *Tribolium confusum* (5 LE of *T. confusum* 4th instar larvae per trail). The circular trails were offered to the parasitoids as freshly laid trails (0 h) or 48 h after deposition. Both trail types had been re-dissolved with n-hexane (25 µl) prior to the beginning of the bioassays (N = 28 per time interval). Test circle: hexane-extracted larval host trails. Control circle: hexane only. Mean differences (Δ) of **a)** walked sections and **b)** residence time (indicated by the clock inset) on test and control circle at different time points are displayed. The parasitoid's trail-following activity at different time intervals was analyzed by *Student's t*- test or *Wilcoxon's* rank-sum test (ns = not significant, $P > 0.05$)

efficiently perceivable. Consequently, the CHCs lost the ability to induce trail-following behavior in the parasitoid.

Liquid CHCs are expected to have a greater interorganismic, informative relevance than solid ones because of their higher vapor pressure (Menzel et al. 2019; Othmer and Conwell 1945). Thus, they might not only be perceivable upon contact and bind to the odorant-binding proteins in the olfactory sensilla but could also be detected over some distance via the gas phase. For example, ants can perceive long-chain CHCs for differentiation of nestmates and non-nestmates without physical contact from a distance of 1 cm (Brandstaetter et al. 2008). This indicates that long-chain CHCs are volatile at least to a certain extent. Since *H. sylvanidis* females follow CHC trails of *T. confusum* host larvae by zig-zag movements along the trail, they most probably can directly contact the trail components; however, perception over a short distance cannot be excluded.

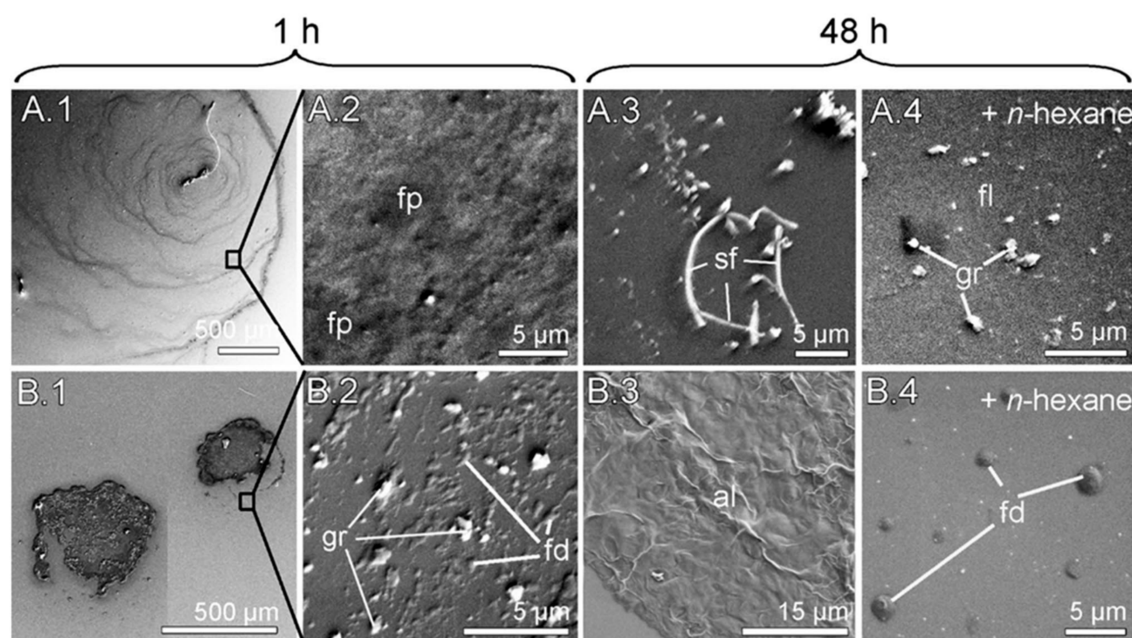

Fig. 5 Cryo-SEM images of non-sterile-filtered hexane extracts of *Tribolium confusum* larvae applied to polar (A.1–4) and apolar (B.1–4) silicon wafers. Photos were taken at different time intervals after release onto the substrate, i.e. after 1 h (A.1–2, B.1–2) and 48 h (A.3–4, B.3–4). Note the "coffee ring" pattern in A.1, thin fluid patches in A.2, solidified filaments in A.3, very thin, re-dissolved fluid patches and granules in A.4, voluminous residues in B.1, tiny fluid droplets at the edges of larger patches in B.2, solidified amorphous films in B.3, and re-dissolved small fluid droplets in B.4. al, solidified, amorphous layer; fd, fluid droplets; fl, fluid layer; fp, fluid patches; sf, solidified filaments; gr, granules

Due to their melting behavior, some mono- and dimethyl-branched CHCs are expected to be liquid in freshly laid larval trails at ambient temperatures, whereas other monomethyl-branched alkanes might begin to solidify right after trail deposition (Brooks et al. 2015; Gibbs and Pomonis 1995). The liquid aggregate state of methyl-branched alkanes might be relevant for eliciting trail-following behavior in *H. sylvanidis*. This suggestion is supported by our previous study, which showed that methyl-branched CHCs on the cuticle of *T. confusum* larvae are exploited by *H. sylvanidis* females for host recognition when directly contacting the host larvae (Awater-Salendo et al. 2020).

The sterile-filtered extracts were lacking some of those methyl-branched alkanes that were present in low quantities in the non-sterile trails, i.e. 3-MeC28, 5-MeC29, 5,X-DiMeC29, and 3,X-DiMeC29. We might have lost the low quantities of these compounds and *n*-C30 when filtering the larval extract through a sterile membrane. Despite the presence of these five compounds in non-sterile trails 24 h and 48 h after trail deposition, the kairomonal activity of these trails decreased significantly over time compared to freshly laid trails (= 0 h), and almost no trail-following behavior by parasitoid females was observed after 48 h. This suggests that these five long-chained CHCs are either not relevant in eliciting trail-following behavior of *H. sylvanidis* or they are no longer in a liquid phase at these time intervals past trail deposition, and thus not perceivable for the parasitoid.

Does also the substrate onto which *T. confusum* host larvae deposit their trails, affect the persistence of trail kairomonal activity? In the present study, we investigated host trails by applying larval cuticular extracts on inert glass ground, a polar substrate. This approach enabled us to determine possible shifts in the CHC profile over time since trails could be extracted from the glass for chemical analysis after distinct time intervals past deposition. Glass as substrate is very different from the natural substrates onto which *T. confusum* larvae release their trails. Larvae of this species are living where grains and further processed or refined plant and food products stored by humans are available. Thus, they release trails both onto polar substrates such as fine flour with all its carbohydrates, but also on whole grains with their often waxy, apolar surface. Our previous studies showed that the kairomonal activity of *T. confusum* trails naturally laid by larvae on course wheat grist (including seed coats, broken grain kernels) persisted as long as those trails extracted with

hexane, i.e. 48 h past trail deposition (Fürstenau and Hilker 2017). Larval trails released by other insect species (coccinellid larvae, caterpillars) on natural substrates (leaves) are also known to elicit behavioral responses by braconid parasitoid species only for a maximum of two days past deposition. As in our study, these trails exclusively consist of long-chain CHCs with different structural features (Nakashima et al. 2004; Rostás and Wölfling 2009).

Under natural conditions, abiotic environmental factors might significantly influence the speed of transition from the liquid to solid state of CHCs. Especially temperature can significantly affect this transition (Gibbs 2002; Gibbs and Pomonis 1995). For instance, the different CHC profiles of several ant species became entirely liquid at temperatures ranging between 30 °C and 45 °C (Menzel et al. 2019). Hence, it is likely that high temperatures retard the solidification process of CHCs of *T. confusum* larvae. If so, the larval trails would remain longer in the liquid state, and thus their kairomonal activity for parasitoids might last longer. Low temperatures, in contrast, have an opposite effect and accelerate solidification of CHCs. Accelerated solidification *T. confusum* CHCs possibly driven by low temperatures is expected to result in an earlier loss of the kairomonal activity of trails within two days at room temperature. We suggest that differences in ambient temperature lead to different behavioral responses of parasitoids to host trails consisting of CHCs.

If the loss of the informative activity of insects CHCs is caused by the solidification of the CHC blend, as suggested above for *T. confusum* trails, the question arises what keeps CHCs liquid or in a liquid-solid phase on the insect cuticle so that they are released in this physical state onto a substrate. Our study suggests that compounds not detectable in hexane extracts of insect CHCs are relevant for the physical state of CHCs on the insect's cuticle. Proteins in an aqueous medium are candidate compounds. They are known to function as CHC carriers, transporting the apolar CHCs through an aqueous medium from their synthesis site (e.g., the oenocytes) to the outer layer of the cuticle (Mohammadzadeh-K et al. 1969; Schal et al. 1998; Wigglesworth 1990). In addition, it is known for insect footprints, i.e. adhesion-mediating tarsal secretion, that they are formed by nanodroplets containing an apolar and a polar phase (Betz 2003; Hasenfuss 1977; Vötsch et al. 2002). The apolar phase often consists of a chemical blend whose hydrocarbon composition is often similar to that of the epicuticular grease, whereas water-soluble carbohydrates and proteins are assumed to be components of the polar phase (Geiselhardt et al. 2009, 2010; Gerhardt et al. 2016; Vötsch et al. 2002). The presence of proteinaceous components has been confirmed for tarsal fluids by Vötsch et al. (2002) and Betz et al. (2016). Hence, proteins might be involved in processes, which prevent CHCs from adopting a folded conformation and from forming densely packed assemblages by intermolecular interactions. After deposition of a blend of CHCs and proteins onto a substrate, proteins would quickly denature, thus allowing the CHCs to interact. To further elucidate the cause of the loss of the kairomonal activity of larval trails, future studies should focus on both the polar and apolar chemical composition of the epicuticular grease of host larvae and their naturally laid trails.

Taken together, our study showed that the CHC blend of an insect trail does neither qualitatively nor quantitatively change within 48 h past trail deposition but loses its kairomonal activity for a parasitoid within this time interval. This result was independent of whether microbial degradation of trails had been excluded or not. Since a change in the CHC composition of the trail cannot be made responsible for the temporary kairomonal effect, our study rather suggests that CHCs present in their liquid phase in freshly laid host trails gradually form solid structures and thus become less perceptible (as host-indicating cues) for foraging parasitoids. Future studies need to further elucidate whether as yet unconsidered polar compounds contribute to the gradual loss of kairomonal activity of host larval CHC trails.

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Data Availability Datasets generated in the current study are available upon request via the corresponding author.

Code Availability Coding in "R" used in the current study is available upon request via the corresponding author.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Additional Declarations for Articles in Life Science Journals that Report the Results of Studies Involving Humans and/or Animals Not applicable.

Conflicts of Interest/Competing Interests The authors have no conflicts of interest to declare that are relevant to the contents of this article.

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Supporting data

The following supplemental data is available for this article:

Tables

Table S1 Results of the SIMPER analysis comparing the chemical composition of sterile-filtered hexane extracts of *Tribolium confusum* larvae 0 h, 24 h or 48 h after trail (extract) deposition

Table S2 Results of the SIMPER analysis comparing the chemical composition of non-sterile-filtered hexane extracts of *Tribolium confusum* larvae 0 h, 24 h or 48 h after trail (extract) deposition

Table S3 Comparison of the chemical composition of sterile-filtered and non-sterile-filtered hexane extracts of Tribolium confusum larvae 0 h, 24 h or 48 h after trail (extract) deposition

Table S4 Behavioral response of female *Holepyris sylvanidis* to trails consisting of non-sterile-filtered hexane extracts of *Tribolium confusum* larvae; the response to trails was tested **a)** 0 h, 24 h, and 48 h after trail deposition (no further treatment of trails), or **b)** after application of *n*-hexane to freshly laid trails (0 or 48 h-old trails (re-dissolved larval trails) just prior to testing

Table S1 Results of the SIMPER analysis comparing the chemical composition of sterile-filtered hexane extracts of *Tribolium confusum* larvae 0 h, 24 h or 48 h after trail (extract) deposition (overall average dissimilarity index = 5.35)

Compound	RI_{cal}[a]	Average dissimilarity	Contribution (%)	Cumulative (%)	Hours after trail deposition[b]		
					0	24	48
n-C27	2700	1.48	27.71	27.71	45.10	45.50	46.70
n-C25	2498	1.09	20.29	48.01	17.20	17.10	17.60
n-C29	2897	0.63	11.79	59.80	9.99	10.00	9.71
11-/13-MeC27	2730	0.42	7.80	67.60	6.95	6.91	6.91
5-MeC27	2747	0.26	4.79	72.38	3.28	3.25	3.11
12-/13-/14-MeC28	2828	0.21	3.86	76.24	0.81	1.02	0.56
n-C28	2797	0.20	3.82	80.06	4.11	4.11	4.03
3.X-DiMeC27	2803	0.18	3.29	83.35	2.28	2.04	1.97
5.X-DiMeC27	2778	0.16	2.95	86.30	1.69	1.64	1.50
11-/13-MeC29	2928	0.14	2.61	88.92	1.07	1.06	0.78
n-C26	2598	0.12	2.22	91.14	2.58	2.45	2.48
4-MeC28	2854	0.10	1.89	93.03	0.47	0.46	0.30
3-MeC27	2771	0.09	1.75	94.78	2.97	2.94	2.89
3-MeC29	2970	0.07	1.27	96.05	0.20	0.23	0.27
10-/11-/12-/13-MeC26	2633	0.06	1.05	97.09	0.46	0.44	0.41
3-MeC25	2573	0.05	1.00	98.09	0.33	0.33	0.32
4-MeC26	2656	0.05	0.85	98.94	0.23	0.21	0.18
n-C31	3096	0.02	0.44	99.39	0.12	0.07	0.08
11-/13-MeC25	2533	0.02	0.41	99.80	0.11	0.12	0.12
5-MeC25	2550	0.01	0.20	100.00	0.07	0.07	0.08
			99.99	100.00	100.02	99.95	100.00

[a] Retention index calculated on a HP-5ms capillary column (30 m x 0.25 mm x 0.5 µm)

[b] Relative quantities are given as % LE^{-1}, *N* = 5 per time interval

Table S2 Results of the SIMPER analysis comparing the chemical composition of non-sterile-filtered hexane extracts of *Tribolium confusum* larvae 0 h, 24 h or 48 h after trail (extract) deposition (overall average dissimilarity index = 4.68)

Compound	RI_{cal}[a]	Average dissimilarity	Contribution (%)	Cumulative (%)	Hours after trail deposition[b]		
					0	24	48
n-C27	2708	1.04	23.55	23.55	31.50	31.30	30.70
11-/13-MeC27	2735	0.58	13.06	36.61	8.68	8.86	8.62
n-C25	2503	0.48	10.88	47.48	11.30	11.40	11.30
5-MeC27	2750	0.41	9.37	56.85	4.20	4.24	4.85
n-C29	2904	0.29	6.51	63.36	16.20	16.50	16.30
11-/13-MeC29	2930	0.22	4.95	68.31	2.49	2.38	2.40
n-C28	2801	0.19	4.30	72.61	5.84	6.03	5.99
5,X-DiMeC27	2781	0.13	2.84	75.45	2.09	2.03	1.97
3-MeC27	2774	0.13	2.83	78.29	4.07	4.21	4.13
n-C26	2599	0.11	2.45	80.74	2.90	2.95	3.01
3,X-DiMeC27	2806	0.10	2.29	83.02	2.83	2.82	2.82
12-/13-/14-MeC28	2830	0.09	2.11	85.14	1.09	1.09	1.09
10-/11-/12-/13-MeC26	2633	0.09	1.95	87.08	0.80	0.80	0.88
3-MeC28	2873	0.07	1.51	88.60	0.39	0.26	0.33
4-MeC28	2856	0.06	1.31	89.91	0.83	0.76	0.79
3,X-DiMeC29	3004	0.06	1.30	91.21	0.38	0.32	0.36
n-C31	3098	0.06	1.29	92.50	0.73	0.68	0.70
4-MeC26	2658	0.05	1.24	93.74	0.44	0.39	0.48
11-/13-MeC25	2534	0.05	1.12	94.86	0.31	0.27	0.37
3-MeC25	2573	0.05	1.05	95.92	0.73	0.71	0.75
3-MeC29	2972	0.04	0.98	96.90	0.78	0.76	0.79
5-MeC29	2948	0.04	0.96	97.86	0.50	0.41	0.41
5,X-DiMeC29	2979	0.04	0.90	98.76	0.46	0.38	0.41
5-MeC25	2550	0.03	0.71	99.48	0.20	0.18	0.22
n-C30	2999	0.02	0.52	100.00	0.35	0.32	0.34
			100.01		100.09	100.05	100.01

[a] Retention index calculated on a HP-5ms capillary column (30 m x 0.25 mm x 0.5 µm)

[b] Relative quantities are given as % LE^{-1}, *N* = 6 per time interval

Table S3 Comparison of the chemical composition of sterile-filtered and non-sterile-filtered hexane extracts of *Tribolium confusum* larvae 0 h, 24 h or 48 h after trail (extract) deposition

			Hours after trail deposition[c]								
			0[d]			**24**[d]			**48**[d]		
			Non-sterile	**Sterile**		**Non-sterile**	**Sterile**		**Non-sterile**	**Sterile**	
Compound[a]	**ID**	**RI$_{cal}$**[b]	Mean ± SE (ng)	Mean ± SE (ng)	***P***[e]	Mean ± SE (ng)	Mean ± SE (ng)	***P***[e]	Mean ± SE (ng)	Mean ± SE (ng)	***P***[e]
n-C25	I	2498	20.12 ± 0.67	20.52 ± 3.04	ns	19.47 ± 0.77	17.99 ± 3.53	ns	19.10 ± 0.49	21.83 ± 2.40	ns
11-/13-MeC25	II	2533	0.56 ± 0.06	0.13 ± 0.03	***	0.46 ± 0.03	0.13 ± 0.03	***	0.63 ± 0.10	0.15 ± 0.03	**
5-MeC25	III	2550	0.36 ± 0.04	0.08 ± 0.02	***	0.31 ± 0.02	0.08 ± 0.02	***	0.38 ± 0.06	0.10 ± 0.01	**
3-MeC25	IV	2573	1.31 ± 0.12	0.40 ± 0.09	***	1.22 ± 0.06	0.36 ± 0.10	***	1.28 ± 0.07	0.40 ± 0.08	***
n-C26	V	2598	5.19 ± 0.23	3.10 ± 0.43	**	5.02 ± 0.15	2.61 ± 0.54	**	5.12 ± 0.15	3.07 ± 0.34	***
10-/11-/12-/13-MeC26	VI	2633	1.44 ± 0.16	0.53 ± 0.04	**	1.37 ± 0.09	0.46 ± 0.11	***	1.50 ± 0.15	0.51 ± 0.09	***
4-MeC26	VII	2656	0.79 ± 0.08	0.27 ± 0.03	***	0.66 ± 0.05	0.21 ± 0.06	***	0.82 ± 0.10	0.22 ± 0.05	***
n-C27	VIII	2700	56.50 ± 2.77	53.27 ± 5.70	ns	53.37 ± 1.68	46.66 ± 7.15	ns	52.23 ± 0.78	57.52 ± 5.41	ns
11-/13-MeC27	IX	2730	15.75 ± 1.56	8.10 ± 0.75	**	15.13 ± 0.90	7.25 ± 1.51	**	14.72 ± 0.95	8.61 ± 1.12	**
5-MeC27	X	2747	7.61 ± 0.65	3.81 ± 0.32	***	7.24 ± 0.34	3.35 ± 0.65	***	8.30 ± 1.19	3.84 ± 0.45	**
3-MeC27	XI	2771	7.30 ± 0.30	3.50 ± 0.39	***	7.17 ± 0.10	3.07 ± 0.59	**	7.02 ± 0.16	3.58 ± 0.38	***
5,X-DiMeC27	XII	2778	3.79 ± 0.35	1.95 ± 0.15	**	3.47 ± 0.19	1.71 ± 0.37	**	3.36 ± 0.18	1.86 ± 0.27	***
n-C28	XIII	2797	10.45 ± 0.41	4.86 ± 0.53	***	10.28 ± 0.18	4.23 ± 0.71	***	10.18 ± 0.21	4.97 ± 0.50	***
3,X-DiMeC28	XIV	2803	5.10 ± 0.35	2.64 ± 0.18	***	4.82 ± 0.16	2.15 ± 0.49	**	4.80 ± 0.23	2.42 ± 0.31	**
12-/13-/14-MeC28	XV	2828	1.98 ± 0.19	0.93 ± 0.07	**	1.86 ± 0.12	1.07 ± 0.32	*	1.87 ± 0.18	0.70 ± 0.14	***
4-MeC28	XVI	2854	1.50 ± 0.12	0.54 ± 0.03	***	1.29 ± 0.05	0.47 ± 0.13	***	1.35 ± 0.11	0.36 ± 0.07	***
3-MeC28	XVII	2873	0.71 ± 0.16			0.44 ± 0.02			0.57 ± 0.08		
n-C29	XVIII	2897	29.10 ± 1.57	11.77 ± 1.28	***	28.06 ± 0.52	10.42 ± 1.86	***	27.68 ± 0.33	11.96 ± 1.20	***
11-/13-MeC29	XIX	2928	4.51 ± 0.43	1.25 ± 0.12	***	4.06 ± 0.32	1.15 ± 0.29	***	4.10 ± 0.39	0.93 ± 0.09	**
5-MeC29	XX	2948	0.91 ± 0.11			0.70 ± 0.02			0.71 ± 0.05		
3-MeC29	XXI	2970	1.40 ± 0.10	0.23 ± 0.05	***	1.29 ± 0.05	0.27 ± 0.11	**	1.34 ± 0.09	0.31 ± 0.06	***
5,X-DiMeC29	XXII	2979	0.83 ± 0.05			0.65 ± 0.03			0.70 ± 0.06		
n-C30	XXIII	2999	0.64 ± 0.03			0.54 ± 0.03			0.58 ± 0.05		
3,X-DiMeC29	XXIV	3004	0.68 ± 0.07			0.55 ± 0.04			0.61 ± 0.12		
n-C31	XXV	3096	1.29 ± 0.04	0.13 ± 0.01	**	1.15 ± 0.10	0.08 ± 0.04	***	1.19 ± 0.05	0.10 ± 0.01	***

[a] *n*-alkanes were identified by comparing RIs and mass spectra with authentic standards. Methyl-branched alkanes were tentatively identified by the diagnostic ions, which resulted from favored fragmentation at branched points (see (Fürstenau and Hilker 2017) and by comparing RIs with data from literature

[b] Retention index calculated on a HP-5ms capillary column (30 m x 0.25 mm x 0.5 µm)

[c] For the preparation of host larval trails, see experimental part

[d] N = 5-6 per trail type and time interval

[e] For each compound, the *p*-value denotes significantly quantitative differences between non-sterile and sterile-filtered larval CHC trails of *T. confusum* 0 h, 24 h or 48 h after trail deposition (*Student's t*-test, *Welch's t*-test or *Wilcoxon's* rank-sum test, ns = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Table S4 Behavioral response of female *Holepyris sylvanidis* to trails consisting of non-sterile-filtered hexane extracts of *Tribolium confusum* larvae; the response to trails was tested **a)** 0 h, 24 h, and 48 h after trail deposition (no further treatment of trails), or **b)** after application of *n*-hexane to freshly laid trails (0 or 48 h-old trails (re-dissolved larval trails) just prior to testing. CHC test trail: host larval extracts. Control trail: hexane only. The trail-following activity was evaluated based on (i) the residence time and (ii) the walked sections on a circular trail

Trails: Non-sterile filtered larval extracts of *T. confusum* (5 LE per trail)[a]	**Time interval** (h)	**Residence time**[b]		**Walked sections**[b]	
		CHC trail [c] Mean ± SE (s)	**Control trail** [c] Mean ± SE (s)	**CHC trail** [c] Mean ± SE (sections)	**Control trail** [c] Mean ± SE (sections)
a) Larval trails	0	67.36 ± 4.99[a]	14.72 ± 1.18[b]	5.39 ± 0.46[a]	1.14 ± 0.18[b]
	24	47.75 ± 4.89[a]	8.63 ± 1.08[b]	2.71 ± 0.33[a]	0.86 ± 0.18[b]
	48	28.62 ± 2.56[a]	9.14 ± 1.01[b]	1.25 ± 0.20[a]	0.82 ± 0.14[a]
b) Re-dissolved larval trails	0	73.16 ± 5.84[a]	11.46 ± 1.15[b]	6.21 ± 0.43[a]	1.04 ± 0.22[b]
	48	65.43 ± 5.43[a]	12.68 ± 1.17[b]	4.61 ± 0.36[a]	1.14 ± 0.20[b]

[a] For preparation of host larval trails, see experimental part

[b] Different letters indicate that the parasitoid's response to the control and CHC (test) trail differed significantly ($P < 0.001$, *Student's t*-test or *Wilcoxon's* signed-rank test for paired data)

[c] $N = 28$ per treatment and time interval

Chapter 3:

The importance of methyl-branched cuticular hydrocarbons for successful host recognition by the larval ectoparasitoid *Holepyris sylvanidis*

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The Importance of Methyl-Branched Cuticular Hydrocarbons for Successful Host Recognition by the Larval Ectoparasitoid *Holepyris sylvanidis*

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Abstract
Cuticular hydrocarbons (CHCs) of host insects are used by many parasitic wasps as contact kairomones for host location and recognition. As the chemical composition of CHCs varies from species to species, the CHC pattern represents a reliable indicator for parasitoids to discriminate host from non-host species. *Holepyris sylvanidis* is an ectoparasitoid of beetle larvae infesting stored products. Previous studies demonstrated that the larval CHC profile of the confused flour beetle, *Tribolium confusum*, comprises long chain linear and methyl-branched alkanes (methyl alkanes), which elicit trail following and host recognition in *H. sylvanidis*. Here we addressed the question, whether different behavioral responses of this parasitoid species to larvae of other beetle species are due to differences in the larval CHC pattern. Our study revealed that *H. sylvanidis* recognizes and accepts larvae of *T. confusum*, *T. castaneum* and *T. destructor* as hosts, whereas larvae of *Oryzaephilus surinamensis* were rejected. However, the latter species became attractive after applying a sample of *T. confusum* larval CHCs to solvent extracted larvae. Chemical analyses of the larval extracts revealed that CHC profiles of the *Tribolium* species were similar in their composition, while that of *O. surinamensis* differed qualitatively and quantitatively, i.e. methyl alkanes were present as minor components on the cuticle of all *Tribolium* larvae, but were absent in the *O. surinamensis* CHC profile. Furthermore, the parasitoid successfully recognized solvent extracted *T. confusum* larvae as hosts after they had been treated with a fraction of methyl alkanes. Our results show that methyl alkanes are needed for host recognition by *H. sylvanidis*.

Keywords Bethylidae · GC-MS analysis · Host recognition · Methyl alkanes · Stored product pest · Tenebrionidae · *Tribolium*

Introduction

Cuticular hydrocarbons (CHCs) are present on the surface of all insects and have several important functions, ranging from protection against desiccation to mediation of intra- and inter-specific communication (Gibbs 1998; Ingleby 2015; Menzel et al. 2017; Otte et al. 2018). CHCs play a key role in the chemical communication of many insect species, serving as contact sex pheromones and kairomones for recognition of gender, caste, nest mates, mutualistic partners, and hosts (Howard and Blomquist 2005). The information conveyed by CHCs is based on various CHC types, which may be linear or methyl branched, saturated or unsaturated hydrocarbons with different chain lengths. The CHC patterns vary from species to species; some of these patterns are simple and contain only a few compounds, while others are composed of highly complex multi-component blends with up to 100 compounds (Blomquist and Bagnères 2010).

Despite the complexity of insect CHC profiles, many studies pointed out that in general only a small number of components are involved in the intra- and interspecific communication of insects. For some species, single compounds of a specific CHC blend are sufficient to elicit behavioral responses (Colazza et al. 2007; Guédot et al. 2009; Kühbandner et al. 2012b; Rutledge et al. 2009), whereas for others a combination of several CHCs is required (Sakata et al. 2017; Spikes et al. 2010; Sugeno et al. 2006; Würf et al. 2020). A few

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studies have demonstrated the behavioral activity of straight chain (linear) alkanes and alkenes (Colazza et al. 2007; Dani et al. 2005; Ginzel et al. 2003b). In many cases, however, methyl alkanes have turned out to be the behavior inducing cues (e.g. Guédot et al. 2009; Ginzel et al. 2003a; Lacey et al. 2008; Ruther et al. 2011; Sakata et al. 2017; Silk et al. 2011).

Several species-specific CHCs have already been identified to be used by parasitic wasps to discriminate between mating partners or host species (Ablard et al. 2012; Kühbandner et al. 2012b). For generalist parasitoids, it is assumed that their foraging behavior is mediated by general cues released by different hosts (Steidle and Van Loon 2003), but only little is known about chemical cues used by parasitoid species that attack a broader range of host species.

The polyphagous, cosmopolitan ectoparasitoid *Holepyris sylvanidis* (Bréthes 1913) (Hymenoptera: Bethylidae) has been described to parasitize larvae of various beetle taxa (i.e. Cucujidae, Silvanidae, Tenebrionidae) infesting processed plant products (Amante et al. 2017b; Evans 1969; Hagstrum and Subramanyam 2009). Adults and larvae of these beetles are major pests of stored grain or wheat flour and occur worldwide in warehouses and in the food-processing industry (Hertlein et al. 2011; Vassilakos et al. 2012).

In a previous study we showed that *H. sylvanidis* responds to larval CHCs of its preferred host, the confused flour beetle *Tribolium confusum* (Du Val 1863) (Coleoptera: Tenebrionidae). The parasitoid uses the CHCs of the host larvae for host finding at short range by following CHC trails laid by larvae on the substrate (Fürstenau and Hilker 2017). The CHC profiles of some other potential host species of *H. sylvanidis* have also been chemically analyzed in the past (Baker et al. 1978; Howard et al. 1995; Lockey 1978), but their influence on host recognition or host selection of parasitoids has not yet been examined. Therefore, it is still unknown, whether *H. sylvanidis* recognizes suitable host larvae by a CHC pattern, which different host species have in common.

To address this question, we selected three beetle species which have been described as potential host species of *H. sylvanidis*; (i) *T. confusum*, (ii) the red flour beetle *T. castaneum* (Herbst 1797) (Coleoptera: Tenebrionidae) and (iii) the sawtoothed grain beetle, *Oryzaephilus surinamensis* (Linnaeus, 1758) (Coleoptera: Silvanidae). In addition to the aforementioned study by Fürstenau and Hilker (2017), several previous studies have shown that *T. confusum* and the closely related species *T. castaneum* are accepted as hosts by *H. sylvanidis* (Amante et al. 2017a, 2018; Ahmed et al. 1997; Fürstenau et al. 2016). Because of co-occurrence of *H. sylvanidis* and *O. surinamensis* in storage facilities (e.g. Eliopoulos et al. 2002) this grain beetle has been also considered a potential host species (Amante et al. 2017b; Hagstrum and Subramanyam 2009). However, it is unknown as yet, whether *H. sylvanidis* indeed accepts this species as host and whether the parasitoid can successfully develop on this species. Moreover, the destructive flour beetle, *Tribolium destructor* (Uyttenboogaart 1934) (Coleoptera: Tenebrionidae), a distantly related species of *T. confusum* and *T. castaneum*, was also included as a fourth species in the investigation without ever being described as a host of *H. sylvanidis*. However, it is interesting to note that adults of all three *Tribolium* spp. release 1-pentadecene, a volatile compound, which is considered to be part of the flour beetle aggregation pheromone (Arnaud et al. 2002; Verheggen et al. 2007) and has also been demonstrated to attract *H. sylvanidis* (Fürstenau et al. 2016).

The aim of the present study was to answer the following questions: (i) Do *H. sylvanidis* females recognize and accept larvae of different stored product pest beetle species as hosts? (ii) Does the larval CHC profile of a successfully recognized host species differ from that of a non-host? (iii) Which compounds of the CHC profile of a potential host species act as contact cues for successful host recognition by *H. sylvanidis*?

We analyzed hexane extracts of larvae of the aforementioned four beetle species by gas chromatography coupled with mass spectrometry (GC-MS) and compared the CHC profiles. In order to find out, whether methyl alkanes are responsible for host recognition by *H. sylvanidis*, we isolated them from larval extracts of *T. confusum*. Subsequently, the behavioral responses of *H. sylvanidis* to live and dead larvae of the four beetle species, to crude larval extracts of a host or a non-host species and to a fraction of methyl alkanes of the *T. confusum* CHC profile were tested in contact bioassays.

Methods and Materials

Insects The four stored-product pest beetles and potential host species of *H. sylvanidis*, *T. castaneum*, *T. confusum*, *T. destructor*, and *O. surinamensis* were reared in the laboratory of the Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection (Julius Kühn Institute (JKI), Berlin, Germany). The host insects were reared in a climate chamber in permanent darkness at 25 ± 1 °C and 65 ± 5% RH.

The three analyzed *Tribolium* spp. were kept on the same feeding substrate, a mix of finely ground wheat grist (*Triticum aestivum* Linnaeus 1753) and wheat flour (Type 405, Kaufland, Neckarsulm, Germany, mix = 1:1), in 400 ml glass jars. Rearing of *T. confusum* was conducted as described by Fürstenau et al. (2016). The rearing protocol for *T. castaneum* and *T. destructor* was similar, but modified as follows: Two weeks after emergence from the pupal stage, 150 adults of *T. castaneum* and 50 adults of *T. destructor* were taken from the permanent rearing at the JKI and each placed in a glass jar filled with 150 ml of feeding substrate. The adults (males and females) were left in the jars for one week and could mate and oviposit during this time. Thereafter, they were separated from the feeding substrate by sieving (mesh size = 710 µm) and

transferred to another jar filled with fresh feeding substrate. The egg-infested substrate was stored for subsequent larval development. Approximately five weeks after oviposition, *Tribolium* larvae reached the 4th instar level, the (most) preferred host stage for *H. sylvanidis*. For our rearing, we used adult beetles for at maximum two weeks before replacing them by freshly emerged adults from the permanent rearing.

Oryzaephilus surinamensis was reared on coarsely ground wheat grist. Fifty adults (males and females) were kept in a 400 ml glass jar. After mating and oviposition for one week, the adults were removed manually with forceps and placed in a jar filled with new feeding substrate. Beetles were used for at maximum three weeks before they were replaced by new (freshly emerged) ones for the further rearing. The substrate infested with *O. surinamensis* eggs was stored for subsequent larval development. In each jar we additionally placed a tissue paper to provide a good shelter for the latest instar larvae before pupation and to facilitate the removal of *O. surinamensis* larvae for following bioassays and chemical analysis. Larvae were removed approximately four weeks after oviposition when *O. surinamensis* larvae were 4th instars.

The parasitoid species *H. sylvanidis* was reared on *T. confusum* larvae in a climate chamber (25 ± 1 °C, 57 ± 5% RH). Previous studies had shown that experience with host or host-associated products (e.g. host exuviae or feces) just after emergence can induce behavioral changes in the parasitoid, resulting in reinforcement of inherited host preference (Barron 2001). Therefore, unexperienced ("naïve") *H. sylvanidis* females were used in the host recognition bioassays to exclude any biased effects.

To obtain naïve *H. sylvanidis*, one- to seven-day-old females were collected from the permanent rearing at the JKI (Berlin, Germany) and placed individually with one conspecific male in a Petri dish (94 mm diam., 16 mm height). Each Petri dish was provided with approx. 50 *T. confusum* 4th − 5th instars as hosts and 1.8 g ground wheat grist as food for the host larvae. A honey drop was applied onto the inner surface of the Petri dish lid for carbohydrate nutrition. After having located a host larva, the *H. sylvanidis* female paralyzes the host and pulls it to a sheltered site prior to laying its egg onto the host larva. Therefore, five pipette tips (0.1–20 µl, Carl Roth, Karlsruhe, Germany) were offered in each Petri dish as hiding place for paralyzed host larvae. To prevent parasitoids from escaping, Petri dishes were sealed with parafilm. *Tribolium confusum* larvae were taken from our stock culture. The adult parasitoids could mate and oviposit onto the host larvae for one week. Thereafter, they were transferred to a new Petri dish to mate and oviposit for another week before they were replaced by newly emerged ones. Parasitized host larvae were separated from unparasitized ones and placed individually in a Petri dish (5.0 cm diam.). After pupation of the parasitoid larvae (approx. one week after oviposition) we gently removed host exuviae and larval feces of *T. confusum* with a brush. Thus, freshly emerging *H. sylvanidis* adults had no chance to experience chemical cues of their rearing host, *T. confusum.* Naïve *H. sylvanidis* adult progenies emerged four to six weeks after oviposition and could feed on a drop of honey. Newly emerged parasitoids were separated by sex. Unmated, naïve, one- to four-day-old females were used in bioassays, while older females and males were discarded.

Preparation of Crude Larval Extracts from Potential Host Species For chemical analyses of the CHC profiles of potential host species and subsequent contact bioassays, we prepared the following larval extracts: (1) crude extracts from 4th instar larvae of each species (*T. castaneum*, *T. confusum*, *T. destructor* and *O. surinamensis*) for GC-MS analysis, (2) crude extracts from 4th instar larvae of *T. confusum* and *O. surinamensis* for contact bioassays (*dummy test I*, treatments #7,8,11,12 in Table 1), (3) crude extracts from *T. confusum* 4th instar larvae for separation of *n*-alkanes from methyl branched ones and for contact bioassays testing the different CHC samples (*dummy test II*, treatment #15–16 in Table 1).

A previous study by Fürstenau and Hilker (2017) demonstrated that the chemical composition of processed CHC extracts of *T. confusum*, which had been purified via solid-phase extraction, did not differ from crude extracts of the same beetle species. Therefore, we prepared and used only crude larval extracts of the aforementioned beetle species for our chemical analyses and bioassays.

(1) Crude larval extracts of the four potential host beetle species for chemical analysis were prepared by a procedure slightly modified from that described by Fürstenau and Hilker (2017). Preliminary tests revealed that only small amounts of compounds can be extracted from the cuticle of *O. surinamensis* larvae. Therefore, crude extracts of *O. surinamensis* larvae ($N = 20$) were prepared by immersing 90 larvae in 120 µl *n*-hexane (analytical purification > 98%, VWR, Radnor, USA) for 10 min at ambient temperature. We concentrated the extract under a gentle stream of nitrogen and stored it at -20 °C for further analysis. The 4th instar larvae of the studied host species differed somewhat in size and weight. For example, larvae of *O. surinamensis* were smaller (approx. 4–5 mm) than those of *Tribolium* spp. (approx. 6–10 mm). To prepare crude larval extracts with comparable larval biomass per solvent, we calculated the mean weight of 10 samples with 90 *O. surinamensis* larvae each (= 59.03 mg ± 0.66). We took this value to determine the number of larvae to be used for the other beetle species (*T. confusum* = 25 larvae, *T. castaneum* = 28 larvae, *T. destructor* = 21 larvae). Thereafter, crude larval extracts of *Tribolium* spp. ($N = 20$ for each species) were prepared as described for those of *O. surinamensis*. To

Table 1 Overview of contact bioassays for analyses of host recognition behavior by *Holepyris sylvanidis* females

Bioassay	#	Potential host species	State[a]	Treatment[b]	N[c]
A) Host recognition — Influence of beetle species	1	*T. castaneum*	L/D	untreated	36
	2	*T. confusum*	L/D	untreated	36
	3	*T. destructor*	L/D	untreated	36
	4	*O. surinamensis*	L/D	untreated	36
B) *Dummy test I* — Influence of host larval CHCs	5	*T. confusum*	D	untreated	30
	6	"	D	extracted†	30
	7	"	D	extracted† + re-applied *O. sur*-larval extract‡	30
	8	"	D	extracted† + re-applied *T. con*-larval extract‡	30
	9	*O. surinamensis*	D	untreated	30
	10	"	D	extracted†	30
	11	"	D	extracted† + re-applied *O. sur*-larval extract*	30
	12	"	D	extracted† + re-applied *T. con*-larval extract*	30
C) *Dummy test II* — Influence of host larval CHC fractions	13	*T. confusum*	D	untreated	40
	14	"	D	extracted†	40
	15	"	D	extracted† + re-applied *T. con*-larval extract — **complete CHC profile**^	40
	16	"	D	extracted† + re-applied **methyl-branched CHC-fraction**^^ of *T. con*-larval extract	40

[a] Live (L) or differntly treated dead (**D**) larvae were offered to parasitoids. Larvae were killed at -20 °C. Behavioral responses and searching time until successful host recognition were recorded for a period of 300 s

[b] Different treatments for dead larvae as test stimuli:

† To remove cuticular hydrocarbons, larvae were extracted for 10 min with *n*-hexane

‡ 2 µl (= ½ larval equivalent, LE) of *T. confusum* (*T. con*) or *O. surinamensis* (*O. sur*) crude larval extract were re-applied to extracted larvae

* 1 µl (= ½ LE) of *T. con* or *O. sur* crude larval extracts were re-applied to extracted larvae

^ 2 µl (= ½ LE) of *T. con* larval extract were re-applied to extracted larvae before fractionation with 5 Å-molecular sieves

^^ 2 µl (= ½ LE) of methyl alkane fraction of *T. con* larval extract were re-applied to extracted larvae after fractionation with 5 Å-molecular sieves

[c] Number of replicates

quantify the amount of host larval CHCs, extracts were re-dissolved in 50 µl *n*-hexane containing 1-eicosene as internal standard (IS, 10.4 ng μl^{-1}, Sigma-Aldrich, Taufkirchen, Germany).

(2) Crude larval extracts for contact bioassays were produced as follows: Ten larvae of *T. confusum* or of *O. surinamensis* were immersed in 100 µl *n*-hexane and removed after 10 min before the supernatant was dried under a gentle stream of nitrogen. For the re-application of CHCs on dead and extracted *T. confusum* larvae, the extracted CHCs were re-dissolved in 40 µl *n*-hexane (treatments #7–8 in Table 1). For re-application of CHCs on dead and extracted *O. surinamensis* larvae, the extracted CHCs were re-dissolved in 20 µl *n*-hexane (treatments #11–12 in Table 1). Since *T. confusum* larvae are larger and thicker than those of *O. surinamensis*, 2 µl of crude larval extract were required to uniformly impregnate a *T. confusum* larva. In contrast, applying 1 µl was enough to evenly cover an *O. surinamensis* larva.

(3) Crude larval extracts of *T. confusum* for fractionation and further contact bioassays with different CHC samples were produced by following a method described by Bello et al. (2015). Approximately 2000 freshly killed *T. confusum* larvae were extracted in 5 ml *n*-hexane for 10 min. The supernatant was concentrated to ca. 1000 µl under a gentle stream of nitrogen. To purify the alkanes (both linear and methyl branched), we loaded the crude extract onto an isolute silica gel column (100 mg, Biotage, Uppsala, Sweden), which had been pre-conditioned by rinsing two times with 1 ml dichloromethane (> 99%, Merck, Darmstadt, Germany) and 1 ml *n*-hexane. The sample was eluted from the column by applying four times 1 ml *n*-hexane.

From this eluate (purified CHCs), we took (i) 450 µl (≈ 200 LE of *T. confusum*) for subsequent contact bioassays and (ii) 100 µl (≈ 44 LE of *T. confusum*) for chemical analysis by GC-MS; both types of samples were stored at -20 °C.

To separate the methyl alkanes from linear ones, the remaining eluate was transferred to a 25 ml vial and concentrated to dryness before being re-dissolved in 5 ml isooctane (Merck, Darmstadt, Germany). We added 100 mg of activated 5 Å-molecular sieves (Sigma-Aldrich, Taufkirchen, Germany) per mg of sample, while the vial was flushed with nitrogen for 5 min. To activate the molecular sieves, they had previously been dried in a muffle furnace at 300 °C for 15 h. In the airtight-sealed vial, the extract was then magnetically stirred at ambient temperature for 18 h. Thereafter, the supernatant containing isolated methyl alkanes was removed and filtered through a Whatman filter paper (9.0 cm diam.). Prior to storage of this supernatant for later contact bioassays, 100 µl (≈ 44 LE of *T. confusum*) were taken for GC-MS analysis. In total, we fractionated three samples of larval extracts ($N = 3$). For chemical analysis, we used 100 µl each of the supernatant containing isolated methyl alkanes and of the eluate containing purified CHCs ($N = 3$ per sample type). Under a gentle stream of nitrogen, we concentrated each sample to dryness before adding 50 µl *n*-hexane containing 1-eicosene (10.4 ng µl^{-1}) as IS.

To test the influence of different structural groups of CHCs on the host recognition behavior of *H. sylvanidis*, we used purified CHCs (mixture of linear and methyl alkanes) as well as isolated methyl alkanes, which were obtained by separation from *T. confusum* crude larval extracts. For treatment #15 (Table 1), we concentrated 46 µl of each sample with purified CHCs (≈ 20 LE of *T. confusum*) under a gentle stream of nitrogen and dissolved each sample in 80 µl *n*-hexane. For treatment #16 (Table 1), we took 50 µl (≈ 20 LE of *T. confusum*) of each sample with isolated methyl alkanes and prepared the extracts as described for those in treatment #15.

GC-MS Analysis of Crude Larval Extracts of Potential Host Species and Different CHC Samples of *T. confusum* Larvae GC-MS analyses of crude larval extracts and different CHC samples of *T. confusum* larval extracts were performed on a GCT Premier – TOF Mass Spectrometer (Waters, Milford, USA) coupled to a GC System 7890A (Agilent Technologies, Waldbronn, Germany). One µl of each sample was injected in splitless mode, keeping the injector at 250 °C with helium as carrier gas (1 ml min^{-1}). The oven temperature program started at 40 °C, which was held for 4 min and increased then at 10 °C min^{-1} to 300 °C. The final temperature was held for 20 min. Samples were separated on a 30 m HP-5MS capillary column (250 µm diam., 0.25 µm film thickness, Agilent JandW Scientific). After a solvent delay of 5 min, masses were scanned every 0.9 s with a range from 50 to 600 *m*/*z* (electronic impact [EI] ionization = 70 eV, source temperature = 230 °C).

For structure assignments of detected compounds, an authentic *n*-alkane standard (*n*-C7-*n*-C40, Sigma-Aldrich, Taufkirchen, Germany) was additionally injected. Linear alkanes were identified by comparing their mass spectra and the calculated retention indices (RIs) with those of the *n*-alkane standard. In contrast, no reference compounds of the methyl alkanes were available to us. For each compound, we tentatively determined the position of its methyl branching based on the characteristic mass spectrometric fragmentation and the calculated RIs according to van den Dool and Kratz (1963). We further compared the RI and the fragmentation pattern with those published by Lockey (1978), Hebanowska et al. (1989, 1990), Howard et al. (1995), Spiewok et al. (2006), Geiselhardt et al. (2009), Svensson et al. (2014), Gerhardt et al. (2016), and Fürstenau and Hilker (2017).

Individual compounds were quantified relative to the peak area of the IS. CHC samples were standardized by calculating the mean amount of each compound (in ng) per one larval equivalent (LE).

Contact Bioassay: Host Finding and Recognition Behavior To analyze the response of one-to four-day-old, naïve *H. sylvanidis* females to 4th instar (live and dead) larvae of (i) *T. confusum*, (ii) *T. castaneum*, (iii) *T. destructor* and (iv) *O. surinamensis*, we performed a series of different contact bioassays (Table 1). The bioassay methods were similar to those described by Fürstenau and Hilker (2017). The parasitoid behavior was observed in a test arena, which consisted of a circle (9.0 cm diam.) drawn on a sheet of white paper and covered by the lid of a plastic Petri dish (94 mm diam., 16 mm height). To avoid any external interference, the test arena was surrounded by a cardboard box (70.5 cm x 44.5 cm x 41 cm). For an even illumination, a strip of light-emitting diodes (λ = 625 nm, Barthelme GmbH & Co, Nürnberg, Germany) was located 5 cm above the box. All bioassays took place at 25 ± 1 °C. Live or differently treated dead larvae of each species were placed individually in the center of the test arena. Beetle larvae were killed by freezing at -20 °C for 2 h and allowed to warm to room temperature for 30 min prior to biotest start. In three different experimental set ups we tested the influence of the following host stimuli on the host recognition behavior of *H. sylvanidis* (Table 1):

A) untreated larvae of the previously mentioned beetle species
B) crude larval extracts (CHC profiles) of *T. confusum* or *O. surinamensis*, respectively, applied onto extracted larvae of these two species
C) different CHC samples of *T. confusum* larvae (including one sample of isolated methyl alkanes) applied onto extracted *T. confusum* larvae

Untreated as well as extracted larvae of the respective species were used as control treatments in bioassays B) and C).

All bioassays were prepared by placing the host stimulus (a single live or dead larva) in the center of the test arena. When a crude larval host extract was applied onto a test larva, the solvent could evaporate for 1 min prior to release of the parasitoid into the arena. A bioassay began by releasing a single *H. sylvanidis* female onto the circle drawn on the sheet of paper lining the arena. The release side of parasitoids rotated clockwise to avoid any biased results due to possible side preference. Each individual parasitoid was observed for max. 300 s. As described by Fürstenau and Hilker (2017), we recorded (i) whether the parasitoid located a host larva and showed host recognition behavior when encountering it and (ii) determined the searching time until successful host recognition. We defined successful host recognition as the moment when *H. sylvanidis* bends its abdomen around the larva to start paralyzing it. Once the parasitoid successfully found and recognized its host, the experiment was stopped. Test individuals, host stimuli, and the paper lining the arena were replaced by new ones after each run. The lid of the Petri dish was cleaned with a 70% ethanol solution (> 96%, Berkel AHK, Ludwigshafen, Germany). When a parasitoid rested more than 50% of the observation time - less than 5% (34 occasions) of all bioassays listed in Table 1 - the individual was not included in the statistical analysis and replaced by a new one, which showed active searching behavior. When live or dead larvae of *Tribolium* spp. and *O. surinamensis* were offered as potential hosts (bioassay "A"), each treatment was replicated 36 times ($N = 36$). When we studied the influence of crude larval extracts of *T. confusum* or *O. surinamensis* on host recognition of *H. sylvanidis* (bioassay "B"), we repeated each treatment 30 times ($N = 30$). When the influence of different CHC samples of *T. confusum* larvae on host recognition of *H. sylvanidis* were tested (bioassay "C"), each treatment was replicated 40 times ($N = 40$).

Bioassay: Host Acceptance and Oviposition To figure out whether the parasitoid accepts larvae of *Tribolium* spp. and *O. surinamensis* for oviposition, we conducted a further bioassay, which additionally allowed us to check the development of the parasitoid´s offspring on the beetle larvae. *Holepyris sylvanidis* deposits a single egg onto a host larva and only a single parasitoid larva can develop per host (Amante et al. 2017a).

A naïve, one-to four-day-old *H. sylvanidis* was offered one live 4th instar larva in a Petri dish (5.0 cm diam.) for a period of 24 h. The potential host larva was provided with 0.4 g finely ground wheat grist. Furthermore, one pipette tip (0.1–20 µl, Carl Roth, Karlsruhe, Germany) was offered as hiding place to the parasitoid since *H. sylvanidis* pulls paralyzed host larvae to a shelter site prior to oviposition. For each potential host species, we tested 40 female parasitoids on 40 host larvae ($N = 40$ per species). After the 24-hour-exposure time to the parasitoid, the parasitized larvae were transferred to a climate chamber (25 ± 1 °C, 57 ± 5% RH, permanent darkness) for further development. Larvae onto which the parasitoid had oviposited, were recognized by the parasitoid's egg on the cuticle of the host larval. Unparasitized larvae were classified as not-accepted hosts. After four weeks, the number of emerging parasitoids per host species were counted (= successful host acceptance). Host larvae, which were not accepted as hosts and those on which *H. sylvanidis* larvae had not completed its development, were counted as failed host acceptance.

Statistical Analysis All statistical analysis were computed in "R", version 3.6.1 (R Core Team 2019), except of the SIMPER analysis, which was performed in "PAST", version 3.26 (Hammer et al. 2001).

For an across-beetle-species-comparison, we prepared data sets obtained by chemical analyses of the CHC profiles as follows. For some peaks, the mass spectrum and RI indicated that several internally branched alkanes (branching at position 10, 11, 12, 13, 14 or 15) co-eluted. The RIs of these compounds differed slightly among the *Tribolium* species due to the different positions of the methyl branching. Therefore, we pooled these internally branched alkanes. Additionally, we only included compounds, which were present in more than 50% of all extracts of a beetle species. When one of the selected compounds was below the detection limit in some extracts of a beetle species, we handled the missing compound as follows. To avoid any bias in the subsequent statistical analysis, we generated a random peak for each missing value by the "rnorm()"-function in "R". Since the missing compound had been detected in other extracts of a beetle species, we selected the smallest peak area, which this compound had in these extracts, as mean and calculated the standard deviation based on the four smallest peak areas. In total, 43 pseudo peaks were generated. Finally, we normalized all selected compounds by calculating the quantitative contribution of each compound to one LE of the beetle species.

To statistically compare the CHC patterns of the tested beetle species, we calculated relative amounts of detected compounds in 1 LE of each beetle species and summed up all amounts to 100%. Based on the Bray-Curtis dissimilarity, a one-way analysis of similarity (ANOSIM) was then performed with 99,999 random permutations using the package "vegan" (version 2.5-6, Oksanen et al. 2019) in "R". The dissimilarity of groups is stated by the *R*-value, ranging between 0 and 1. An *R*-value close to 1 indicates a clear discrimination, whereas an *R*-value close to 0 indicates a high similarity (Clarke 1993). We also applied an analysis of similarity percentages (SIMPER) to identify compounds, which contribute the most (i) to the dissimilarity between the CHC profiles of *Tribolium* spp. or (ii) to the dissimilarity between the CHC profiles of *Tribolium* spp. and *O. surinamensis*. The differences among CHC profiles were visualized by performing a non-metric multidimensional scaling (NMDS) calculated on Bray-Curtis dissimilarity. The stress value associated with

NMDS indicates how close the algorithm of NMDS fits to the used data set. A NDMS with a stress value < 0.1 indicates a good fit of the NMDS ordination or low data distortion (Clarke 1993; Dexter et al. 2018).

A comparison of the behavioral responses of *H. sylvanidis* to the offered different host stimuli was based on (i) the host recognition rate per potential host species/treatment and (ii) the mean searching time until successful host recognition of the offered larva. The host recognition rate was analysed by the test of equality of proportions followed by a pairwise comparison of proportions with *Bonferroni-Holm* correction (Newcombe 1998a, b). When the parasitoid's response to dead, differently treated *O. surinamensis* larvae (treatments #9–12 in Table 1) was tested in contact bioassays, results were evaluated by *Fisher's* exact test, followed by a pairwise comparison of proportions with *Bonferroni-Holm* correction. Since the *Shapiro-Wilk* test of normality revealed that the mean searching times required by the parasitoids were not normally distributed in all treatments, we applied the *Kruskal-Wallis* test for comparing the mean searching time of parasitoids exposed to different host stimuli. Thereafter, differences in the mean searching time between the different treatments were pairwise compared using *Wilcoxon* rank sum test with *Bonferroni-Holm* correction.

To analyze the host acceptance behavior of *H. sylvanidis*, we determined for each species the number of larvae successfully accepted as hosts and of those that were not. We recorded "successful host acceptance", when parasitoid offspring emerged from the host and "failed host acceptance", when host larvae were left unparasitized or when parasitoid larvae could not successfully develop inside the host. Finally, the proportions of successful and failed host acceptance were analyzed by the test of equality of proportions followed by a *Bonferroni-Holm* corrected pairwise comparison of proportions across the beetle species.

Results

Comparison of Larval CHC Profiles of Possible Host Species

Our GC-MS analysis revealed that crude larval extracts of *O. surinamensis* and *Tribolium* spp. exclusively consist of saturated cuticular hydrocarbons (CHCs) with chain lengths from *n*-C25 to *n*-C36. In total, we identified 31 compounds, including 12 *n*-alkanes, 14 monomethyl alkanes and five dimethyl alkanes (Table 2; Fig. 1a, b). The CHC profiles showed quantitative and qualitative differences between beetle species, but a common pattern within the genus *Tribolium* was observed (Table 2).

In *Tribolium* spp., a series of *n*-alkanes with chain lengths from *n*-C25 to *n*-C33 represented the dominating group of substance in quantities of 93% (*T. confusum*), 90% (*T. castaneum*), and 79% (*T. destructor*). Most prominent components were *n*-C27 (*T. confusum* and *T. destructor*, entry 10) and *n*-C29 (*T. castaneum*, entry 22). *n*-Alkanes with more than 30 carbon atoms (entries 25, 26, 27) accounted for less than 10% of all CHC profiles analyzed from larvae of this genus. In addition to the fraction of *n*-alkanes, various monomethyl- and dimethyl alkanes were identified in larval extracts of the *Tribolium* spp. Based on the position of the methyl branch, monomethyl alkanes were either internally branched (branching at positions 10, 11, 12, 13, 14, 15 – since positional isomers could not be separated, co-occurrence is indicated by a slash) or externally branched (branching at position 3, 4 or 5), but both groups were present in all *Tribolium* extracts. Dimethyl alkanes were most abundant in the extracts of *T. destructor* (entries 5, 15, 17, 21), while only one dimethyl alkane was present in *T. confusum* (entry 15) and *T. castaneum* (entry 13). The mass spectra of two dimethyl alkanes (5,X-DiMeC27 and 3,X-DiMeC28; entries 15 and 17) did not allow a clear determination of the position of the second methyl group. Therefore, the unknown position was labelled by 'X' (Table 2). Overall, seven methyl alkanes were found in all extracts of *Tribolium* spp. (3-MeC25, 11-/13-MeC27, 3-MeC27 and 12-/13-/14-MeC28; entries 4, 11, 14 and 18) (Table 2). Among these compounds, 11-MeC27 and 13-MeC27 were most abundant (*T. confusum* = 3%, *T. castaneum* = 6%, *T. destructor* = 7%).

In contrast to the CHC profiles of *Tribolium* spp., the profile of *O. surinamensis* was less diverse and contained only linear alkanes, but no methyl alkanes. In total, the assigned peaks represented *n*-alkanes with chain lengths from *n*-C25 to *n*-C36 (Table 2; Fig. 1a). The most abundant component was *n*-C31 (entry 26), and compounds with more than 29 carbon atoms contributed to 77% of the total peak area (Table 2).

Comparison of the CHC profiles of *Tribolium* spp. larvae among each other and with those of *O. surinamensis* larvae by an ANOSIM showed a significant dissimilarity among the beetle species ($R = 0.9988$, $P < 0.001$), which were clearly separated in the NDMS plot (Fig. 2). *Tribolium* spp., however, clustered closer together, indicating that the composition of their CHC profiles was more similar compared to *O. surinamensis*. This was confirmed by the overall average dissimilarity index calculated in SIMPER (Table S1); this index was smaller when comparing the *Tribolium* spp. among each other (*T. castaneum* vs. *T. confusum* vs. *T. destructor* = 38.96) than in the pairwise comparison between each *Tribolium* spp. and *O. surinamensis* (*T. castaneum* vs. *O. surinamensis* = 68.75, *T. confusum* vs. *O. surinamensis* = 68.58, *T. destructor* vs. *O. surinamensis* = 79.79, Table S2, S3, S4). In addition, the SIMPER analysis revealed that all compounds, which accounted most for the dissimilarity between *Tribolium* spp. as well as between each *Tribolium* spp. and *O. surinamensis*, were *n*-alkanes (> 10%). When considering the within-genus comparison of *Tribolium* spp., *n*-C25 (entry 1), *n*-C27 (entry 10), and *n*-C29 (entry 22) contributed together by 73% to the observed dissimilarity (Table S1).

Table 2 Cuticular hydrocarbons identified from crude larval extracts of *Tribolium confusum*, *T. castaneum*, *T. destructor* and *Oryzaephilus surinamensis*

				T. confusum[e]		*T. castaneum*[e]		*T. destructor*[e]		*O. surinamensis*[e]	
No.[a]	Compound[b]	RI_{cal}[c]	RI_{lit}[d]	Mean ± SE (ng)	(%)	Mean ± SE (ng)	(%)	Mean ± SE (ng)	(%)	Mean ± SE (ng)	(%)
1	*n*-C25	2498	2500	3.35 ± 0.39	4.40	2.15 ± 0.20	3.11	16.76 ± 2.11	24.04	0.07 ± 0.02	1.90
2	11-/13-MeC25	2533	2534			0.05 ± 0.00	0.07	0.08 ± 0.01	0.11		
3	5-MeC25	2548	2550					0.36 ± 0.04	0.55		
4	3-MeC25	2572	2571	0.04 ± 0.00	0.05	0.27 ± 0.03	0.38	0.43 ± 0.05	0.64		
5	5,11-DiMeC25	2581	2577					0.32 ± 0.03	0.50		
6	*n*-C26	2597	2600	0.67 ± 0.05	0.87	0.53 ± 0.03	0.78	1.89 ± 0.15	2.83	0.02 ± 0.00	0.65
7	10-/11-/12-MeC26	2634	2632	0.09 ± 0.01	0.11	0.10 ± 0.01	0.15				
	12-/13-MeC26							0.32 ± 0.03	0.48		
8	4-MeC26	2656	2656	0.03 ± 0.00	0.03			0.13 ± 0.01	0.20		
9	3-MeC26	2674	2673			0.13 ± 0.02	0.18	0.25 ± 0.03	0.37		
10	*n*-C27	2700	2700	40.37 ± 4.36	49.55	19.13 ± 0.95	28.00	25.06 ± 1.53	38.63	0.16 ± 0.02	4.51
11	11-/13-MeC27	2731	2731	2.28 ± 0.18	2.98	4.32 ± 0.29	6.29	4.75 ± 0.33	7.30		
12	5-MeC27	2748	2750	0.58 ± 0.06	0.72			0.80 ± 0.06	1.22		
13	11,15-DiMeC27	2758	2762			0.35 ± 0.03	0.51				
14	3-MeC27	2771	2773	1.16 ± 0.13	1.39	1.04 ± 0.07	1.52	1.52 ± 0.11	2.28		
15	5, X-DiMeC27	2778	2781	0.35 ± 0.04	0.43			1.95 ± 0.13	3.01		
16	*n*-C28	2797	2800	5.84 ± 0.68	7.04	3.24 ± 0.19	4.71	1.78 ± 0.11	2.87	0.13 ± 0.01	3.46
17	3,X-DiMeC28	2802	2807					2.15 ± 0.12	3.37		
18	12-/13-/14-MeC28	2830	2831	0.21 ± 0.02	0.26	0.20 ± 0.02	0.29	0.33 ± 0.02	0.51		
19	4-MeC28	2855	2856	0.09 ± 0.01	0.11	0.03 ± 0.00	0.05				
20	3-MeC28	2874	2872			0.00 ± 0.00	0.00				
21	4,14-DiMeC28	2886	2893					0.10 ± 0.01	0.16		
22	*n*-C29	2899	2904	19.92 ± 2.96	22.71	30.36 ± 1.67	44.29	1.66 ± 0.08	2.73	0.45 ± 0.03	12.50
23	11-/13-MeC29	2928	2931	0.33 ± 0.03	0.41						
	11-/13-/15-MeC29					0.35 ± 0.03	0.51				
	11-/15-MeC29							0.18 ± 0.01	0.28		
24	3-MeC29	2970	2978	0.22 ± 0.03	0.26	0.33 ± 0.02	0.47				
25	*n*-C30	2995	3000	1.33 ± 0.13	2.01	1.51 ± 0.11	2.22	1.22 ± 0.11	2.05	0.48 ± 0.05	12.55
26	*n*-C31	3094	3100	2.02 ± 0.17	2.85	2.26 ± 0.19	3.29	1.50 ± 0.14	2.52	0.97 ± 0.08	26.04
27	*n*-C32	3194	3200	1.19 ± 0.15	1.85	1.23 ± 0.13	1.80	1.23 ± 0.12	2.08	0.60 ± 0.08	14.60
28	*n*-C33	3295	3300	1.46 ± 0.16	1.96	0.97 ± 0.10	1.40	0.76 ± 0.08	1.27	0.49 ± 0.08	11.66
29	*n*-C34	3395	3400							0.29 ± 0.06	6.52
30	*n*-C35	3495	3500							0.18 ± 0.04	3.95
31	*n*-C36	3595	3600							0.08 ± 0.02	1.66
	Total			81.63 ± 8.53	99.99	68.52 ± 3.57	100.01	65.59 ± 3.60	100.00	3.93 ± 0.42	100.00

Mean amounts (ng ± SE $larva^{-1}$) and relative quantities (% in 1 larval equivalent, LE) are given

[a] Peak numbers refer to Fig. 1a, b

[b] For the identification procedure see experimental part

[c] RI_{cal} = Retention index calculated on a HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm)

[d] RI_{lit} = Retention index as reported for compounds analyzed on HP-5MS or similar columns in the database (http://www.pherobase.com/) and by Fürstenau and Hilker (2017) or others (peak 5 in Hebanowska et al. (1989, 1990) and Svensson et al. (2014); peak 9 in Gerhardt et al. (2016); peak 13 in Spiewok et al. (2006); peak No. 21 in Geiselhardt et al. (2009)). The provided literature RI values of dimethyl alkanes (entries 15 and 17) refer to unambiguously identified compounds described as 5,13-diMeC27 and 3,13-diMeC27

[e] For the preparation of extracts see experimental part

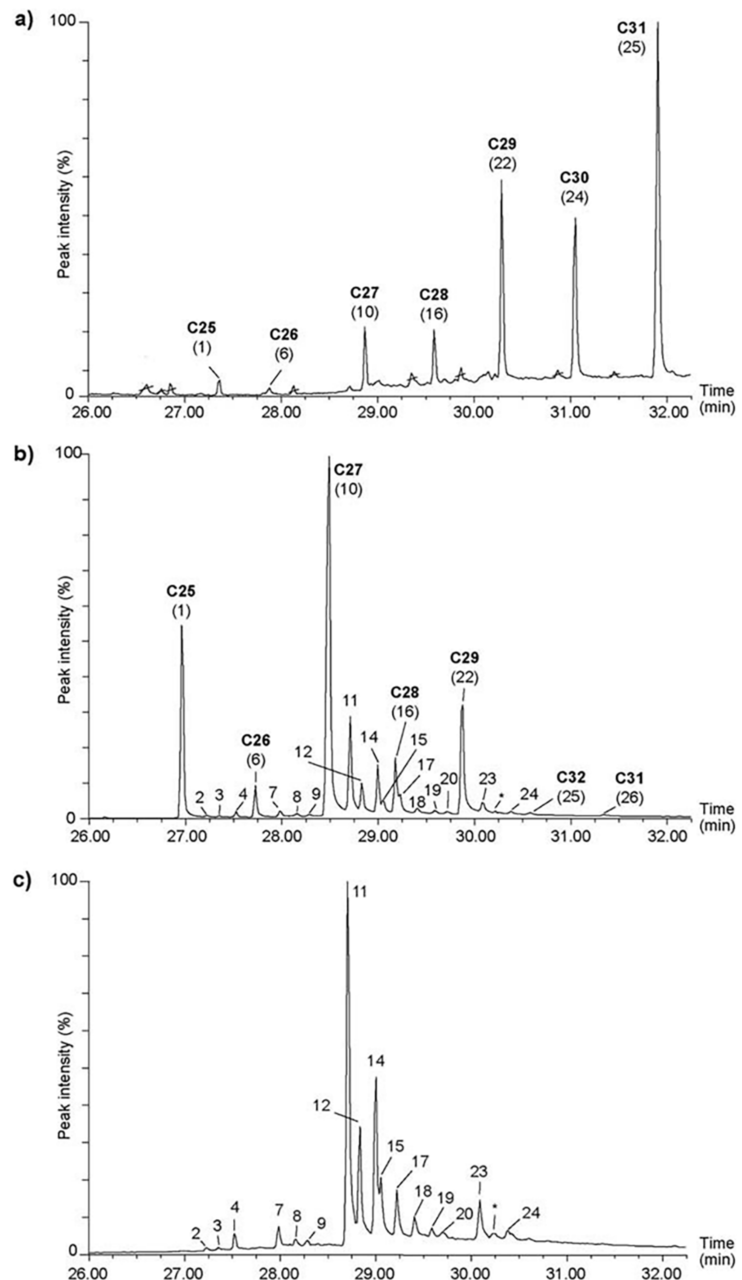


Fig. 1 Partial total ion chromatograms (TIC) of **a**) an un-fractionated *O. surinamensis* crude larval extract, **b**) an un-fractionated *T. confusum* crude larval extract (representative for all three *Tribolium* species as the CHC profiles were similar in their composition with respect to the presence of *n*-alkanes and methyl alkanes) and **c**) the fraction of methyl alkanes of fractionated *T. confusum* crude larval extract. Numbers above peaks represent the CHCs listed in Table 2 and Table S5. The *n*-alkanes (*n*-C25 – *n*-C31) detected in the extracts of *T. confusum* and *O. surinamensis* are highlighted in bold. Crossed-out compounds are contaminations

Fractionation of *T. confusum* larval extracts resulted in a clear separation of linear *n*-alkanes on the one hand and monomethyl and dimethyl alkanes on the other (Fig. 1b, c). All methyl alkanes, which had been previously detected in the un-fractionated crude larval extracts, were recovered after fractionation, but in smaller amounts (Table S5). Furthermore, five compounds were detected in the fraction of methyl alkanes (entries 2, 3, 17, 20 and *), which were not found by our GC-MS analysis of whole *T. confusum* crude larval extracts (Table 2).

Host Finding and Recognition To test whether the parasitoids' host recognition behavior is mediated by compounds present in the larval CHC profile of all possible host species, we conducted several contact bioassays (Table 1).

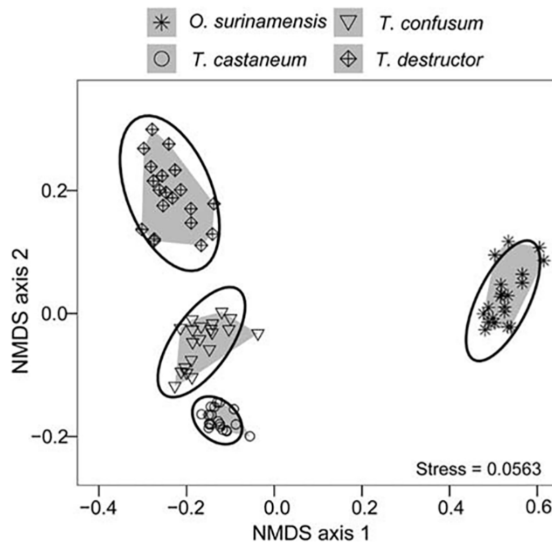


Fig. 2 Comparison of larval CHC profiles of *T. castaneum*, *T. confusum*, *T. destructor* and *O. surinamensis* ($N = 20$ per species) visualized by a non-metric multi-dimensional scaling (NMDS) calculated for two dimensions. The ellipses show the 95%-confidence areas around each centroid for each species

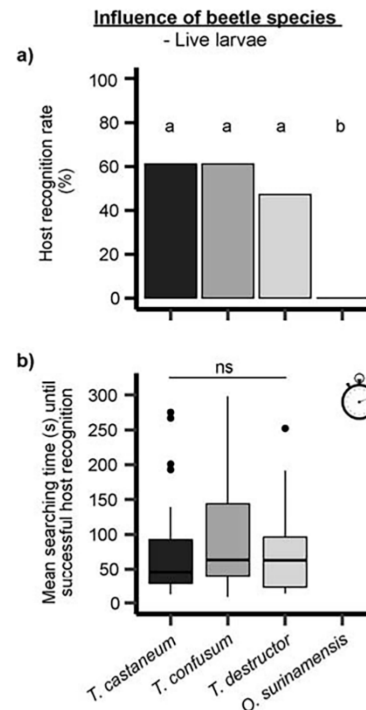
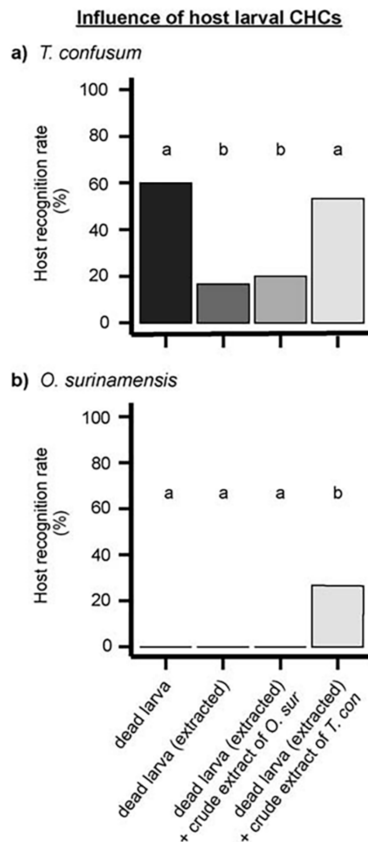


Fig. 3 Behavioral responses of female *H. sylvanidis* in contact bioassays to live larvae of potential host species (*T. castaneum*, *T. confusum*, *T. destructor* and *O. surinamensis*; $N = 36$ per species, max. observation time = 300 s). **a)** Host recognition rate (100% ≙ 36 successful host recognition events per beetle species) was analyzed by the test for equality of proportions followed by pairwise comparison of proportions with *Bonferroni-Holm* correction. Different letters indicate significant differences at $P < 0.05$. **b)** Mean searching time until successful host recognition was analyzed for *Tribolium* spp. and not for *O. surinamensis* as larvae of the latter species were rejected as potential hosts for the parasitoid. Statistical analysis was performed by *Kruskal-Wallis* test (ns = not significant, $P > 0.05$)

When live larvae of different beetle species were offered, *H. sylvanidis* recognized more than 60% of the *T. castaneum* and *T. confusum* larvae as hosts (Fig. 3a). Although the host recognition rate of *T. destructor* larvae was lower (47%), it did not differ significantly among *Tribolium* spp., but was significantly higher compared to the rate of *O. surinamensis* larvae ($\chi^2 = 37.173$, df = 3, $P < 0.001$). In contrast, the host recogntion rate decreased in all *Tribolium* spp. when dead larvae were tested (Figure S2a). Nevertheless, the proportion of successfuly recognized host larvae was significantly higher in *Tribolium* spp. than in *O. surinamensis* ($\chi^2 = 30.424$, df = 3, $P < 0.001$). Live and dead *O. surinamensis* larvae were not recognized as hosts (Fig. 3a, S2a).

Holepyris sylvanidis tended to locate live larvae of *T. castaneum* (Figs. 3b and 80.6 ± 17.3 s) and *T. destructor* (79.4 ± 16.8 s) faster than those of *T. confusum* (102.8 ± 18.4 s). However, the mean searching times until successful host recognition were not significantly different ($\chi^2 = 1.42$, df = 2, $P = 0.49$). When dead larvae were offered, the mean searching time increased in all *Tribolium* spp. (Figure S2b, *T. castaneum* = 115.3 ± 21.3 s, *T. confusum* = 134.1 ± 21.1 s, *T. destructor* = 109.3 ± 20.7 s), but did not differ significantly among them ($\chi^2 = 0.66$, df = 2, $P = 0.72$).

Based on these results, we selected *T. confusum* as representative host species for *Tribolium* spp. and *O. surinamensis* as non-host species for subsequent contact bioassays to investigate the influence of different larval CHC profiles on the host recognition behavior of *H. sylvanidis* (Table 1). Parasitoid females responded significantly different to the four treatments of *T. confusum* larvae (Fig. 4a, $\chi^2 = 19.164$, df = 3, $P < 0.001$; treatments #5 8 in Table 1). While 60% of untreated larvae were successfully recognized as hosts, larvae became significantly less attractive for the parasitoid, when CHCs had been removed by solvent extraction (17%). Applying a CHC extract of *O. surinamensis* larvae onto previously extracted *T. confusum* larvae, led to a slightly increased amount of successfully recognized host larvae (20%). However, this number of successfully recognized larvae did not significantly differ from the one recorded in the previous treatment with extracted larvae. In contrast, the attractiveness of extracted *T. confusum* larvae was restored after applying a CHC extract of *T. confusum* larvae (53%, Fig. 4a). Furthermore, *O. surinamensis* larvae became only attractive to parasitoid females after extracting their CHCs and applying a larval extract of *T. confusum* (Fig. 4b, $P < 0.001$; treatments #9 12 in Table 1). No statistical differences in the mean



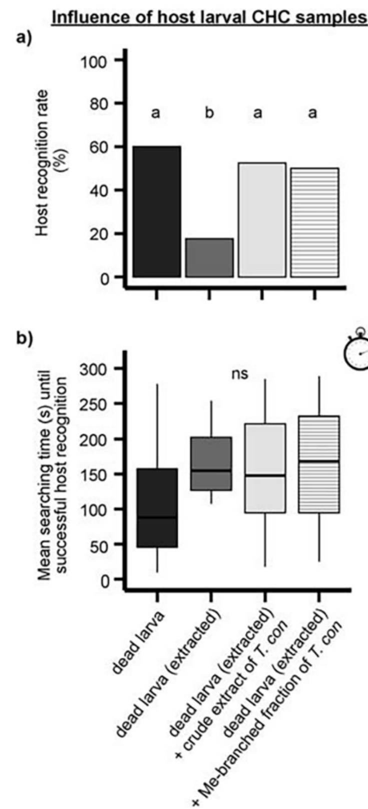


Fig. 4 Host recognition rate in contact bioassays of female *H. sylvanidis* exposed to freshly killed larvae of **a)** *T. confusum* (*T. con*) and **b)** *O. surinamensis* (*O. sur*) treated as follows ($N = 30$ per species and stimulus, 100% ≙ 30 successful host recognition events per stimulus, max. observation time = 300 s): (i) untreated, (ii) extracted with *n*-hexane, (iii) extracted with *n*-hexane and treated with crude larval extract of *O. surinamensis* (½ LE), (iv) extracted with *n*-hexane and treated with crude larval extract of *T. confusum* (½ LE). Host recognition rate was analyzed by the test for equality of proportion (*T. confusum*) or *Fisher's* exact test (*O. surinamensis*) followed by pairwise comparison of proportions with *Bonferroni-Holm* correction. Different letters indicate significant differences at $P < 0.05$

Fig. 5 Behavioral responses of female *H. sylvanidis* to freshly killed *T. confusum* larvae treated as follows ($N = 40$ per stimulus): (i) untreated, (ii) extracted with *n*-hexane, (iii) extracted with *n*-hexane and treated with an un-fractionated larval extract of *T. confusum* (½ LE), (iv) extracted with *n*-hexane and treated with the fraction of methyl alkanes from fractionated *T. confusum* crude larval extract (½ LE). **a)** Host recognition rate (100% ≙ 40 successful host recognition events per stimulus) was analyzed by the test for equality of proportions followed by pairwise comparison of proportions with *Bonferroni-Holm* correction. Different letters indicate significant differences at $P < 0.05$. **b)** Mean searching time until successful host recognition was analyzed by *Kruskal-Wallis* test (ns = not significant, $P > 0.05$)

searching time of *H. sylvanidis* were found between the different combinations of treatments and larvae (Figure S3, $\chi^2 = 4.836$, df = 4, $P = 0.305$).

To elucidate which substance group within the CHC profile of *T. confusum* might be responsible for host recognition of *H. sylvanidis*, different CHC fractions were tested in contact bioassays. Applying the fraction of methyl alkanes onto previously extracted larvae was sufficient to induce host recognition behavior in parasitoid females (Fig. 5a; treatments #13 16 in Table 1). The host recogntion rate (50%) was not significantly different from that of untreated larvae (60%) as well as of larvae, which had been extracted and treated with whole larval extracts (un-fractionated, 53%). In these three treatments significantly more larvae were successfully recognized as hosts compared to the treatment in which previously extracted larvae were offered (18%; $\chi^2 = 17.172$, df = 3, $P < 0.001$). Although *H. sylvanidis* located and recognized untreated *T. confusum* larvae slightly faster than the treated ones, the mean searching time did not differ among treatments #13 16 (Table 1; $\chi^2 = 7.196$, df = 3, $P = 0.07$; Fig. 5b).

Host Acceptance Neither live nor freshly killed *O. surinamensis* larvae had been recognized as hosts in contact bioassays, but we included larvae of this beetle species for control purposes (e.g. to test the possibility that the lack of host recognition response might be due to a short test duration ($t = 300$ s)).

After exposure of larvae to the parasitoid for 24 h, more than 50% of the 40 offered larvae of each *Tribolium* species were parasitized (*T. castaneum* = 29, *T. confusum* = 27, *T. destructor* = 23; Table S6). The parasitoid successfully completed its development from egg to adult on more than 89% of all parasitized host larvae within four weeks (*T. castaneum* = 26, *T. confusum* = 25, *T. destructor* = 22; Table S6). Most parasitoid adults emerged from parasitized *T. castaneum* larvae (hatching rate = 65%), but no significant differences among *Tribolium* spp. were observed (*T. confusum* = 63%, *T. destructor* = 55%; Figure S4). No parasitoid eggs were laid on *O. surinamensis* larvae within a 24 h exposure period and thus, host acceptance of this species significantly differed from those of *Tribolium* spp. ($\chi^2 = 45.624$, df = 3, $P < 0.001$).

Discussion

Our study shows that a larval ectoparasitoid, *H. sylvanidis*, which is supposed to be polyphagous on beetle larvae infesting stored products, selects its hosts by responding to methyl alkanes of the host cuticle. While live and dead 4th instars of the tested *Tribolium* spp. were successfully recognized as hosts by parasitoid females, larvae of *O. surinamensis* were not, although it had been described as host species in the past (Amante et al. 2017b; Evans 1969; Hagstrum and Subramanyam 2009). Chemical analysis of crude larval extracts revealed that the CHC profiles of *Tribolium* spp. and those of *O. surinamensis* varied qualitatively and quantitatively. The cuticular extracts of the four investigated beetle species contained *n*-alkanes. The major difference between the CHC patterns was the presence of methyl alkanes, which represented a minor fraction on the cuticle of *Tribolium* spp., but were absent in *O. surinamensis.* A series of contact bioassays with dead and live larvae of the different beetle species showed that the parasitoid recognizes and accepts a potential host based on the presence of methyl alkanes on the cuticle of an offered larvae. Moreover, a similar response in *H. sylvanidis* was provoked by treating dead, extracted *T. confusum* larvae solely with a fraction of methyl alkanes isolated from *T. confusum* larvae. Therefore, we suggest that host recognition behavior of *H. sylvanidis* is most likely mediated by methyl alkanes from the cuticle of *Tribolium* spp. larvae, whereas the presence of *n*-alkanes alone is not sufficient to elicit host recognition in the parasitoid.

To the best of our knowledge, our results show for the first time that methyl alkanes are essential to elicit host recognition behavior of a parasitoid species. However, methyl alkanes are well known to elicit behavioral responses in sexual communicaton of parasitoid species belonging to the Encrytidae (Ablard et al. 2012) and Pteromalidae (Kühbandner et al. 2012b; Ruther et al. 2011; Steiner et al. 2005, 2007) here the methyl alkanes serve as contact sex pheromones. A recent study by Würf et al. (2020) of a pteromalid species suggests that cuticular methyl alkanes may also act synergistically with *n*-alkanes as contact sex pheromone. For a broad range of non-parasitic insect taxa, methyl alkanes have also been described as contact pheromones, among them ants, beetles, thrips, and psyllids (De Narbonne et al. 2016; Ginzel et al. 2003a; Guédot et al. 2009; Holman et al. 2010; Lacey et al. 2008; Olaniran et al. 2013; Silk et al. 2011; Spikes et al. 2010; Sugeno et al. 2006). For instance, it has been shown that ants use methyl alkanes to discriminate between a mutualistic partner, aphid species, and a non-partner (Sakata et al. 2017).

To study how different larval CHC profiles and isolated methyl alkanes influence host recognition behavior of *H. sylvanidis*, we used solvent extracted *T. confusum* larvae as dummy larvae as (negative) controls. Some of these larvae were still successfully recognized as hosts by the parasitoid in spite of having been extracted leaving no CHCs. This result suggests that not the entire CHC blend was extracted and small amounts of some cuticular lipids may have been left on the larval cuticle. Hence, we cannot fully exclude that some remaining CHCs (e.g. *n*-alkanes) and/or compounds other than CHCs might also affect the behavior of the parasitoid.

However, we observed that parasitoid females responded to solvent-extracted *T. confusum* larvae treated with isolated methyl alkanes similarly as to dead non-extracted *T. confusum* larvae with a complete blend of CHCs.

These results demonstrate the relevance of methyl alkanes for host recognition behavior of *H. sylvanidis.* So far, we cannot pinpoint yet, which methyl alkanes are most important. Neither do we know whether compounds with higher boiling points than those detected here are relevant for the parasitoids' host foraging behavior. The CHC profiles of the closely related *Tribolium* spp. were slightly different. Nevertheless, larvae of each of these beetle species were successfully recognized and accepted as hosts for oviposition by *H. sylvanidis*, and the parasitoid progenies emerged successfully from most of the parasitized host larvae. These findings suggest that host foraging *H. sylvanidis* females rely on those methyl-branched host CHCs, which the analyzed *Tribolium* spp. have in common.

We detected 2 externally methyl-branched alkanes (3-MeC25 and 3-MeC27) and 7 internally methyl-branched alkanes (11-/13-MeC25, 11-/13-MeC27 and 12-/13-/14-MeC28), which were present in all *Tribolium*-CHC profiles; among these compounds, 11-/13-MeC27 and 3-MeC27 belonged to the most abundant methyl alkanes in the *Tribolium*-CHC profiles. These common methyl alkanes might be key components eliciting host recognition behavior in *H. sylvanidis* (entries 2, 4, 11, 14 and 18 in Table 2 and Table S5). This is supported by other studies, which also found behavioral functions for some of these compounds. In particular, 11-/13-MeC27 and 3-MeC27 are known to act as

chemical cues affecting various behaviors in different insects, for example mate finding, (nest) mate and kin recognition or brood discrimination (Guédot et al. 2009; Kühbandner et al. 2012b; Salazar et al. 2015; Silk et al. 2011; Sugeno et al. 2006). Both compounds were also detected in traces laid by *T. confusum* larvae, which elicited trail-following behavior in *H. sylvanids* females (Fürstenau and Hilker 2017). Further studies must be undertaken to investigate, whether single key components are sufficient alone or in combination to elicit host recogntion behavior of *H. sylvanidis*. It will be necessary to carry out similar bioassays with synthetic methyl alkanes to determine those that are essential for host recognition in *H. sylvanidis*.

Moreover, the chirality of methyl CHCs should be considered since previous studies have shown that enantiomers of a certain compound and its racemate may evoke different behavioral responses in parasitoids (Silk et al. 2011; Würf et al. 2020). For instance, males of the egg parasitoid *Ooencyrtus kuvanae* were highly attracted to a blend of 5*S*-MeC27 and (5*R*,17*S*)-DiMeC27, whereas they were repelled by a blend of 5*R*-MeC27 and (5*R*,17*R*)-DiMeC27 (Ablard et al. 2012).

Larvae of *O. surinamensis* have previously been described as potential host of *H. sylvanidis* since both the parasitoid and beetle species have been found at the same storage facilities (e.g. Eliopoulos et al. 2002). However, the chemically mediated parasitoid-host interaction had not been investigated prior to our study, although the CHC profiles of *O. surinamensis* larvae and adults have been examined (Howard et al. 1995). In this study, a different and more diverse composition of CHCs was described compared to our analyses. Besides several *n*-alkanes with a chain length from *n*-C21 to *n*-C35, which represented the predominant group of compounds in the CHC profiles, the authors also identified few alkenes and even some methyl alkanes, which we did not find in our investigation. Possible explanations for the differences in the CHC composition of *O. surinamensis* between the study by Howard et al. (1995) and ours may be the origin of test individuals (different lab strains), different rearing conditions (feeding substrate) and the developmental stage. Howard et al. (1995) did not discriminate between larvae and adults in their analyses of the *O. surinamensis* CHC profiles. Tested insects had been kept on a mix of whole wheat flour enriched with 5% brewer's yeast and rolled oats. In contrast, we used only beetle larvae for our analysis and provided them with coarsely ground wheat grist.

From previous studies it is known that insect CHC profiles may vary depending on strain, age, and diet (see references in Otte et al. 2018; Ngumbi et al. 2020). In particular, changes in the insects' diet may lead to shifts in the composition of CHC profiles since the uptake of amino acids and fatty acids is essential for their biosynthesis, especially that of methyl alkanes (Blomquist and Bagnères 2010; Otte et al. 2014). These diet-induced shifts of CHC compositions can proceed very fast within a short period of one generation or two weeks, thus altering interactions between insects (Geiselhardt et al. 2012; Kühbandner et al. 2012a). Nevertheless, we observed in our contact bioassays that all attempts of *H. sylvanidis* to antennate *O. surinamensis* were aggressively repulsed by the larvae. To exclude this defense behavior, dead larvae were offered as potential hosts to parasitoid females, but they were still not accepted as hosts, most probably due to the lack of methyl alkanes on their cuticle. Therefore, we consider it rather unlikely that *O. surinamensis* is a suitable host for *H. sylvanidis*.

In summary, the present study demonstrates that methyl alkanes present on the cuticle of *Tribolium* larvae mediate host recognition in *H. sylvanidis* and enable parasitoid females to differentiate among host and non-host species. Furthermore, the presence of several identical methyl alkanes in all CHC profiles of *Tribolium* larvae indicates that parasitoid females identify suitable hosts by using these compounds as key substances. Since *Tribolium* spp. are worldwide pests of various stored products, it is very likely that diet-induced shifts in the composition of CHCs can occur. Future systematic studies need to address, how changes in the diet can affect the presence of methyl alkanes on the cuticle of host larva. If a diet shift results in significant changes in the pattern of methyl alkanes of host larvae, further studies should elucidate, how such changes affect host recognition by *H. sylvanidis*.

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Supporting data

The following supplemental data is available for this article:

Tables

Table S1 Results of the SIMPER analysis comparing the larval cuticular hydrocarbons profiles of three *Tribolium* species (*T. castaneum*, *T. confusum* and *T. destructor*)

Table S2 Results of the SIMPER analysis comparing the larval cuticular hydrocarbon profiles of *T. castaneum* and *O. surinamensis*

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Table S5 Mean amounts (ng ± SE larva-1) of cuticular hydrocarbons identified from *T. confusum* larval crude extracts before and after fractionation with 5Å-molecular sieves

Table S6 Number of parasitized larvae of potential host species (*T. castaneum*, *T. confusum*, *T. destructor*, *O. surinamensis*)

Figures

Figure S1 Mass spectra of monomethyl- and dimethyl-branched CHCs identified from host larval crude extracts

Figure S2 Contact bioassay: Behavioral responses of female *H. sylvanidis* in to freshly killed larvae of potential host species

Figure S3 Mean searching time of female *H. sylvanidis* until successful host recognition of freshly killed a) *T. confusum* larvae (different treatments) and b) *O. surinamensis* larvae

Figure S4 Hatching rate of adult *H. sylvanidis* from parasitized beetle larvae

Supplemental data: Tables

Table S1 Results of the SIMPER analysis comparing larval cuticular hydrocarbon profiles of three Tribolium species (*T. castaneum* (*T. cas*), *T. confusum* (*T. con*) and *T. destructor* (*T. des*); overall average dissimilarity index = 38.96)

Compound	**Average dissimilarity**	**Contribution (%)**	**Cumulative (%)**	***T. cas***	***T. con***	***T. des***
n-C29	13.85	35.55	35.55	44.30	22.70	2.72
n-C27	7.45	19.13	54.68	28.00	49.50	38.60
n-C25	7.09	18.21	72.88	3.11	4.40	24.00
11-/13-MeC27	1.58	4.05	76.93	6.29	2.97	7.29
n-C28	1.40	3.59	80.52	4.71	7.03	2.86
3,X-DiMeC28	1.12	2.88	83.40	0.00†	0.00†	3.37
5,X-DiMeC27	1.00	2.21	85.97	0.00†	0.43	3.01
n-C31	0.77	1.99	87.96	3.29	2.85	2.52
n-C26	0.70	1.79	89.75	0.78	0.87	2.83
n-C32	0.63	1.62	91.37	1.80	1.85	2.08
n-C30	0.60	1.54	92.91	2.22	2.01	2.05
n-C33	0.47	1.21	94.13	1.40	1.96	1.27
5-MeC27	0.41	1.05	95.17	0.00†	0.72	1.22
3-MeC27	0.33	0.84	96.02	1.52	1.39	2.27
3-MeC25	0.21	0.55	96.57	0.00†	0.06	0.64
5-MeC25	0.19	0.50	97.06	0.38	0.00†	0.55
11,15-DiMeC27	0.17	0.44	97.50	0.51	0.00†	0.00†
5,11-DiMeC25	0.17	0.43	97.93	0.00†	0.00†	0.50
3-MeC29	0.16	0.41	98.34	0.47	0.28	0.00†
3-MeC26	0.13	0.33	98.67	0.18	0.00†	0.37
10-/11-/12-/13-MeC26*	0.12	0.32	98.98	0.15	0.12	0.48
12-/13-/14-MeC28	0.09	0.24	99.23	0.29	0.26	0.51
11-/13-MeC29*	0.08	0.21	99.44	0.51	0.41	0.29
4-MeC26	0.07	0.18	99.62	0.00†	0.04	0.22
4,14-DiMeC28	0.06	0.15	99.77	0.00†	0.00†	0.17
11-/13-MeC25	0.05	0.14	99.90	0.07	0.00†	0.16
4-MeC28	0.04	0.09	100.00	0.05	0.11	0.00†
3-MeC28	0.00	0.00	100.00	0.00	0.00†	0.00†
n-C36	0.00	0.00	100.00	0.00†	0.00†	0.00†
n-C35	0.00	0.00	100.00	0.00†	0.00†	0.00†
n-C34	0.00	0.00	100.00	0.00†	0.00†	0.00†

* Compounds with similar RI were pooled despite different positions of the methyl group

† Compound is no component in the specific cuticular hydrocarbon profile of the three beetle species. For the SIMPER analysis, the value of this compound was determined as ‘0.00’

Table S2 Results of the SIMPER analysis comparing larval cuticular hydrocarbon profiles of *T. castaneum* (*T. cas*) and *O. surinamensis* (*O. sur*) (overall average dissimilarity index = 68.75)

Compound	Average dissimilarity	Contribution (%)	Cumulative (%)	*T. cas*	*O. sur*
n-C29	15.89	23.11	23.11	44.30	12.50
n-C27	11.75	17.09	40.20	28.00	4.51
n-C31	11.38	16.55	56.75	3.29	26.00
n-C32	6.40	9.31	66.06	1.80	14.60
n-C30	5.17	7.52	73.57	2.22	12.60
n-C33	5.13	7.46	81.03	1.40	11.70
n-C34	3.26	4.74	85.77	0.00[†]	6.52
11-/13-MeC27	3.15	4.58	90.35	6.29	0.00[†]
n-C35	1.97	2.87	93.22	0.00[†]	3.95
n-C25	0.92	1.34	94.56	3.11	1.90
n-C36	0.83	1.21	95.77	0.00[†]	1.67
3-MeC27	0.76	1.11	96.88	1.52	0.00[†]
n-C28	0.72	1.04	97.92	4.71	3.46
11,15-DiMeC27	0.25	0.37	98.29	0.51	0.00[†]
11-/13-MeC29*	0.25	0.37	98.66	0.51	0.00[†]
3-MeC29	0.24	0.34	99.00	0.47	0.00[†]
5-MeC25	0.19	0.27	99.27	0.38	0.00[†]
12-/13-/14-MeC28	0.14	0.21	99.48	0.29	0.00[†]
n-C26	0.13	0.19	99.67	0.78	0.65
3-MeC26	0.09	0.13	99.80	0.18	0.00[†]
10-/11-/12-/13-MeC26*	0.07	0.11	99.91	0.15	0.00[†]
11-/13-MeC25	0.03	0.05	99.96	0.07	0.00[†]
4-MeC28	0.02	0.04	100.00	0.05	0.00[†]
3-MeC28	0.00	0.00	100.00	0.00	0.00[†]
5,X-DiMeC27	0.00	0.00	100.00	0.00[†]	0.00[†]
5-MeC27	0.00	0.00	100.00	0.00[†]	0.00[†]
4,14-DiMeC28	0.00	0.00	100.00	0.00[†]	0.00[†]
5,11-DiMeC25	0.00	0.00	100.00	0.00[†]	0.00[†]
3,X-DiMeC28	0.00	0.00	100.00	0.00[†]	0.00[†]
4-MeC26	0.00	0.00	100.00	0.00[†]	0.00[†]
3-MeC25	0.00	0.00	100.00	0.00[†]	0.00[†]

* Compounds with similar RI were pooled despite different positions of the methyl group

[†] Compound is no component within the specific cuticular hydrocarbon profile of the two beetle species. For the SIMPER analysis, the value of this compound was determined as '0.00'

Table S3 Results of the SIMPER analysis comparing larval cuticular hydrocarbon profiles of *T. confusum* (*T. con*) and *O. surinamensis* (*O. sur*) (overall average dissimilarity index = 68.58)

Compound	**Average dissimilarity**	**Contribution (%)**	**Cumulative (%)**	***T. con***	***O. sur***
n-C27	22.51	32.82	32.82	49.50	4.51
n-C31	11.59	16.91	49.72	2.85	26.00
n-C32	6.38	9.30	59.02	1.85	14.60
n-C30	5.27	7.68	66.71	2.01	12.60
n-C29	5.09	7.43	74.13	22.70	12.50
n-C33	4.85	7.07	81.20	1.96	11.70
n-C34	3.26	4.75	85.96	0.00†	6.52
n-C35	1.97	2.88	88.84	0.00†	3.95
n-C28	1.79	2.61	91.45	7.03	3.46
11-/13-MeC27	1.49	2.17	93.61	2.97	0.00†
n-C25	1.47	2.14	95.75	4.40	1.90
n-C36	0.83	1.21	96.97	0.00†	1.67
3-MeC27	0.70	1.01	97.98	1.39	0.00†
5-MeC27	0.36	0.53	98.51	0.72	0.00†
5,X-DiMeC27	0.22	0.32	98.82	0.43	0.00†
11-/13-MeC29*	0.21	0.30	99.12	0.41	0.00†
n-C26	0.16	0.24	99.36	0.87	0.65
3-MeC29	0.14	0.21	99.57	0.28	0.00†
12-/13-/14-MeC28	0.13	0.19	99.76	0.26	0.00†
10-/11-/12-13-MeC26*	0.06	0.08	99.84	0.12	0.00†
4-MeC28	0.06	0.08	99.92	0.11	0.00†
3-MeC25	0.03	0.05	99.97	0.06	0.00†
4-MeC26	0.02	0.03	100.00	0.04	0.00†
11,15-DiMeC27	0.00	0.00	100.00	0.00†	0.00†
5-MeC25	0.00	0.00	100.00	0.00†	0.00†
4,14-DiMeC28	0.00	0.00	100.00	0.00†	0.00†
3-MeC28	0.00	0.00	100.00	0.00†	0.00†
5,11-DiMeC25	0.00	0.00	100.00	0.00†	0.00†
3-MeC26	0.00	0.00	100.00	0.00†	0.00†
3,X-DiMeC28	0.00	0.00	100.00	0.00†	0.00†
11-/13-MeC25	0.00	0.00	100.00	0.00†	0.00†

* Compounds with similar RI were pooled despite different positions of the methyl group

† Compound is no component within the specific cuticular hydrocarbon profile of the two beetle species. For the SIMPER analysis, the value of this compound was determined as ‘0.00’

Table S4 Results of the SIMPER analysis comparing larval cuticular hydrocarbon profiles of *T. destructor* (*T. des*) and *O. surinamensis* (*O. sur*) (overall average dissimilarity index = 79.79)

Compound	Average dissimilarity	Contribution (%)	Cumulative (%)	*T. des*	*O. sur*
n-C27	17.04	21.36	21.36	38.60	4.51
n-C31	11.76	14.74	36.10	2.52	26.00
n-C25	11.06	13.86	49.96	24.00	1.90
n-C32	6.26	7.85	57.81	2.08	14.60
n-C30	5.25	6.58	64.39	2.05	12.60
n-C33	5.19	6.51	70.89	1.27	11.70
n-C29	4.89	6.13	77.02	2.72	12.50
11-/13-MeC27	3.65	4.57	81.59	7.29	0.00†
n-C34	3.26	4.09	85.67	0.00†	6.52
n-C35	1.97	2.48	88.15	0.00†	3.95
3,X-DiMeC28	1.69	2.11	90.26	3.37	0.00†
5,X-DiMeC27	1.51	1.89	92.15	3.01	0.00†
3-MeC27	1.14	1.43	93.57	2.27	0.00†
n-C26	1.09	1.37	94.94	2.83	0.65
n-C36	0.83	1.04	95.98	0.00†	1.67
n-C28	0.65	0.82	96.80	2.86	3.46
5-MeC27	0.61	0.76	97.56	1.22	0.00†
3-MeC25	0.32	0.40	97.96	0.64	0.00†
5-MeC25	0.28	0.34	98.31	0.55	0.00†
12-/13-/14-MeC28	0.26	0.32	98.63	0.51	0.00†
5,11-DiMeC25	0.25	0.31	98.94	0.50	0.00†
10-/11-/12-/13-MeC26*	0.24	0.30	99.24	0.48	0.00†
3-MeC26	0.19	0.23	99.47	0.37	0.00†
11-/13-MeC29*	0.15	0.18	99.66	0.29	0.00†
4-MeC26	0.11	0.13	99.79	0.22	0.00†
4,14-DiMeC28	0.09	0.11	99.90	0.17	0.00†
11-/13-MeC25	0.08	0.10	100.00	0.16	0.00†
11,15-DiMeC27	0.00	0.00	100.00	0.00†	0.00†
3-MeC29	0.00	0.00	100.00	0.00†	0.00†
3-MeC28	0.00	0.00	100.00	0.00†	0.00†
4-MeC28	0.00	0.00	100.00	0.00†	0.00†

* Compounds with similar RI were pooled despite different positions of the methyl group

† Compound is no component within the specific cuticular hydrocarbon profile of the two beetle species. For the SIMPER analysis, the value of this compound was determined as ‘0.00’

Table S5 Mean amounts (ng ± SE larva^{-1}) of cuticular hydrocarbons identified from *T. confusum* larval crude extracts before and after fractionation with 5Å-molecular sieves

				T. confusum larval extracts[e]	
				Purified CHCs[f]	Methyl alkanes[g]
No.[a]	Compound[b]	RI_{cal}[c]	RI_{lit}[d]	Mean ± SE (ng)	Mean ± SE (ng)
1	*n*-C25	2496	2500	12.19 ± 2.83	
2	11-/13-MeC25	2530	2534	0.08 ± 0.03	0.02 ± 0.01
3	5-MeC25	2548	2550	0.04 ± 0.01	0.02 ± 0.01
4	3-MeC25	2569	2574	0.30 ± 0.07	0.12 ± 0.04
6	*n*-C26	2596	2600	1.65 ± 0.41	
7	10-/11-/12-MeC26	2629	2633	0.28 ± 0.08	0.17 ± 0.06
8	4-MeC26	2653	2658	0.07 ± 0.02	0.04 ± 0.02
10	*n*-C27	2696	2700	28.62 ± 4.02	
11	11-/13-MeC27	2727	2733	5.45 ± 0.88	4.07 ± 0.86
12	5-MeC27	2744	2750	1.43 ± 0.27	0.86 ± 0.25
14	3-MeC27	2767	2773	2.43 ± 0.34	1.46 ± 0.46
15	5,X-DiMeC27	2775	2781	0.75 ± 0.10	0.61 ± 0.08
16	*n*-C28	2793	2800	3.12 ± 0.15	
17	3,X-DiMeC28	2798	2807	0.85 ± 0.06	0.48 ± 0.07
18	12-/13-/14-MeC28	2827	2833	0.26 ± 0.06	0.20± 0.07
19	4-MeC28	2852	2856	0.13 ± 0.01	0.08 ± 0.02
20	3-MeC28	2870	2865	0.07 ± 0.02	0.02 ± 0.01
22	*n*-C29	2894	2900	8.34 ± 0.40	
23	11-/13-MeC29	2925	2931	0.82 ± 0.21	0.44 ± 0.14
*	5-MeC29	2942	2948	0.12 ± 0.07	0.14 ± 0.05
24	3-MeC29	2966	2978	0.27 ± 0.06	0.21 ± 0.08
25	*n*-C30	2994	3000	0.48 ± 0.32	
26	*n*-C31	3090	3100	0.55 ± 0.42	

[a] Peak numbers refer to Figure 1b + c (main text); numbering of peaks relates to the numbering used in Table 2 (main text)

[b] For the identification procedure see experimental part

[c] RI_{cal} = Retention index calculated on a HP-5ms capillary column (30 m x 0.25 mm x 0.25 µm)

[d] RI_{lit} = Retention index as reported for compounds analyzed on HP-5ms or similar columns in the database (http://www.pherobase.com/) and by Fürstenau and Hilker (2017) or others (peak 9 in Gerhardt et al. (2016)). The provided literature RI values of dimethyl alkanes (entries 15 and 17) refer to unambiguously identified compounds described as 5,13-diMeC27 and 3,13-diMeC27

[e] Cuticular extracts of approx. 2000 *T. confusum* larvae were fractionated according to Bello et al. (2015)

[f] For the preparation of samples see experimental part

[g] For the preparation of samples see experimental part

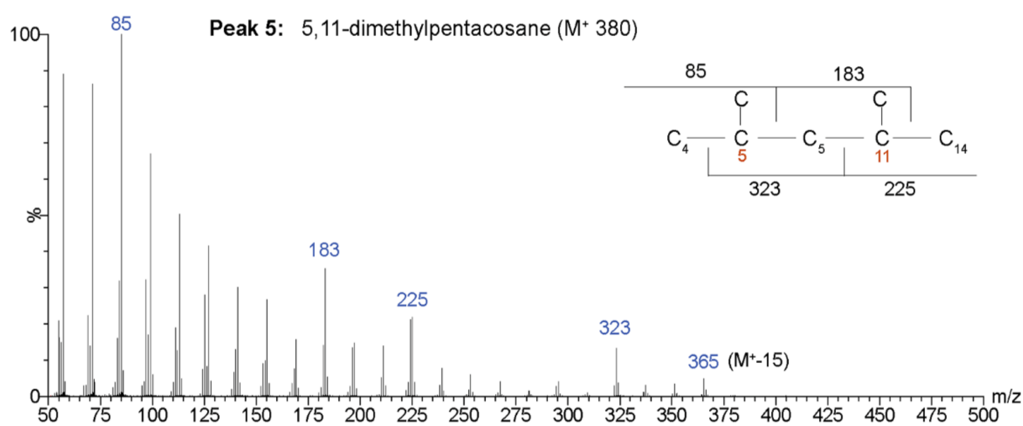
[*] 5-MeC29 was not found in our first GC-MS analysis of crude larval *T. confusum* extracts (see Table 2) but described earlier by Fürstenau and Hilker (2017)

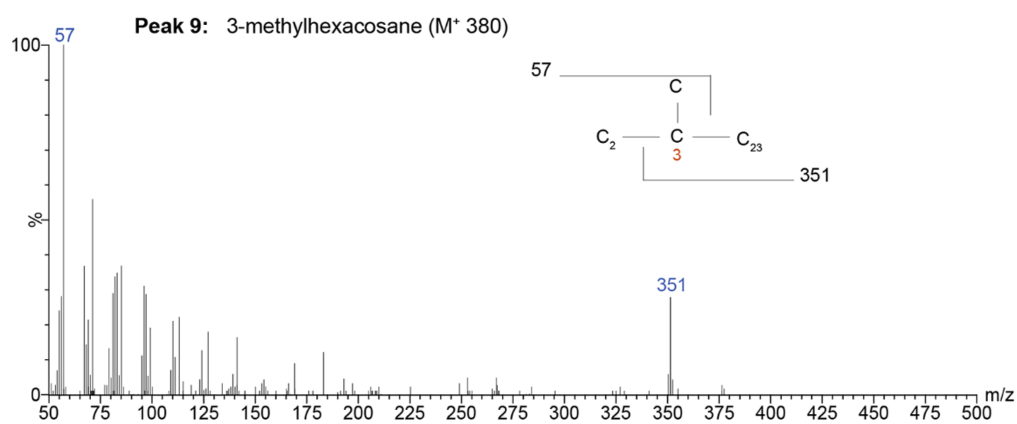
Table S6 Number of parasitized beetle larvae of potential host species (*T. castaneum*, *T. confusum*, *T. destructor*, *O. surinamensis*), which had been offered for 24 h to the parasitoid *H. sylvanidis* in oviposition bioassays, and the number of emerged *H. sylvanidis* adults after four weeks

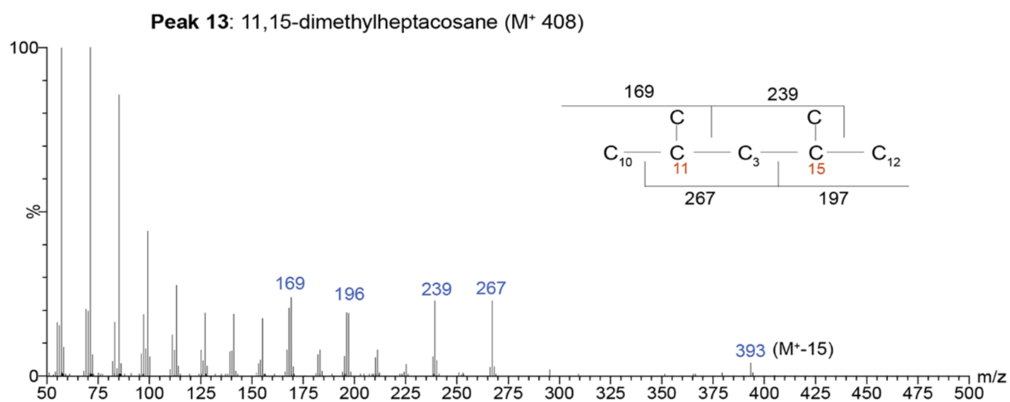
Host species	Number of parasitized beetle larvae (after 24 h) [a]	Number of emerged *H. sylvanidis* adults (after 4 weeks)
T. castaneum	29	26
T. confusum	27	25
T. destructor	23	22
O. surinamensis	0	0

[a] We offered 40 live larvae of each host species (N = 40 per host species) for 24 h to *H. sylvanidis* females for oviposition

Supplemental data: Figures







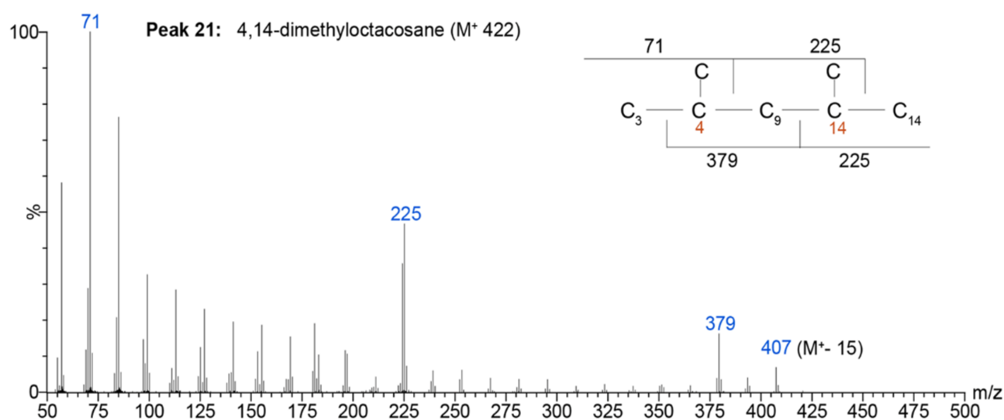


Figure S1 Mass spectra of monomethyl- and dimethyl branched CHCs identified from crude larval extracts of 4th *T. castaneum*, *T. confusum, and T. destructor*. Previously, mass spectra and assignments of branching points (labelling diagnostic ions) of several methyl branched CHCs had been published (Fürstenau and Hilker, 2017). For these CHCs (peak 2, 3, 4, 7, 8, 11, 12, 14, 15, 17, 18, 19, 20, 23, 24 in Table 2, main text) positions of branching points were assigned on the basis of characteristic fragmentation patterns displayed in the mass spectra and comparison with published data. Here we show mass spectra of CHCs (peak 5, 9, 13, and 21 in Table 2, main text), which were not detected previously. Diagnostic signals resulting from fragmentation at branching points and M^+-15 fragments are labelled. Structure assignments are shown in the respective inserts

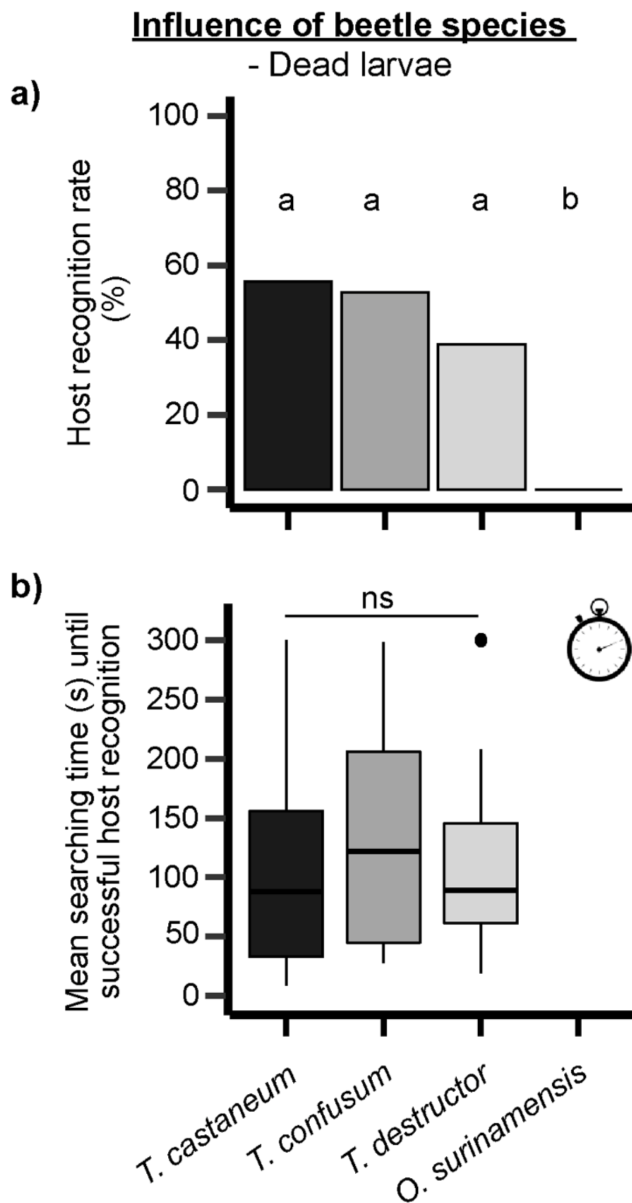


Figure S2 Contact bioassay: Behavioral responses of female *H. sylvanidis* ~~in~~ to freshly killed larvae of potential host species (*T. castaneum*, *T. confusum*, *T. destructor* and *O. surinamensis; N = 36* per species, max. observation time = 300 s). **a)** Host recognition rate (100% ≙ 36 successful host recognition events per beetle species) was analyzed by the test for equality of proportions followed by pairwise comparison of proportions with *Bonferroni-Holm* correction. Different letters indicate significant differences at $P < 0.05$. **b)** Mean searching time until successful host recognition was analyzed for *Tribolium* spp. but not for *O. surinamensis,* as larvae of the latter species were rejected as hosts by the parasitoid. Statistical analysis was performed by *Kruskal-Wallis* test (ns = not significant, $P > 0.05$)

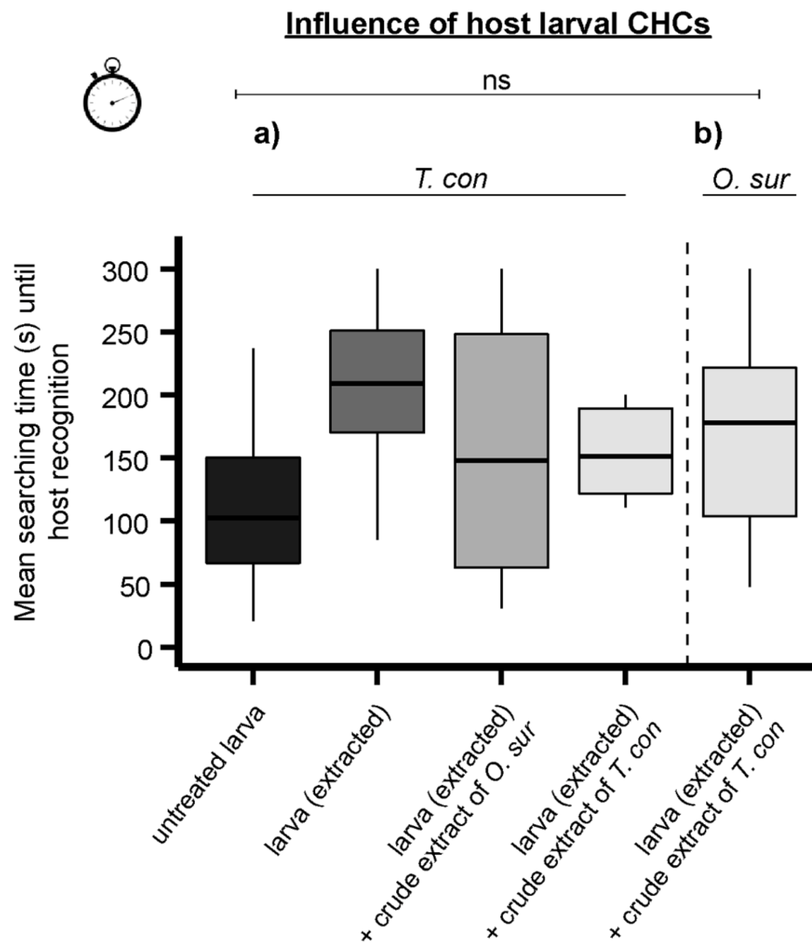


Fig. S3 Mean searching time of female *H. sylvanidis* until successful host recognition of freshly killed **a)** *T. confusum* larvae (*T. con*) treated as follows (*N* = 30 per species and stimulus, max. observation time = 300 s): i) untreated, ii) extracted with *n*-hexane, iii) extracted with *n*-hexane and treated with a larval crude extract of *O. surinamensis* (*O. sur*, ½ LE), iv) extracted with *n*-hexane and treated with a larval crude extract of *T. confusum* (½ LE). **b)** As in the contact bioassays, offering *O. surinamensis* larvae only treatment iv (dead larvae (extracted) + larval crude extract of *T. con*,) elicited successful host recognition behavior in *H. sylvanidis* (see Fig. 4, main text), only for this treatment the mean searching time could be determined and statistically compared by *Kruskal-Wallis* test (ns = not significant, $P > 0.05$)

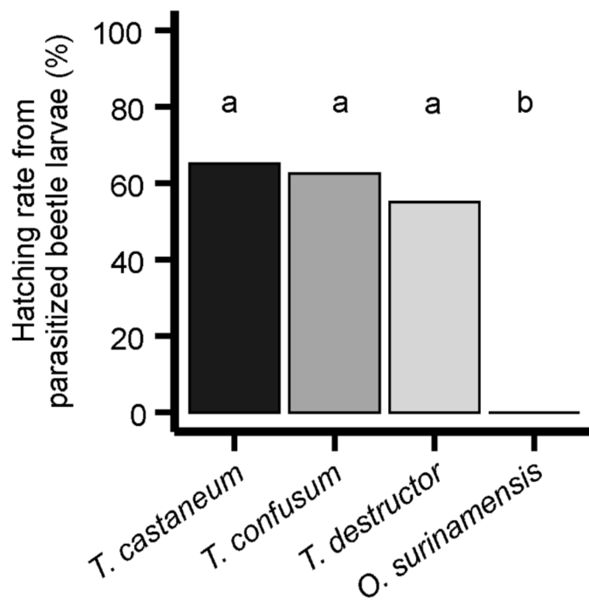


Fig. S4 Hatching rate of adult *H. sylvanidis* from beetle larvae which had been offered as potential host species (*T. castaneum*, *T. confusum*, *T. destructor* and *O. surinamensis*, *N* = 40 per species) to female parasitoids for 24 h in oviposition bioassays. Data were analyzed by the test for equality of proportions followed by pairwise comparison of proportions with *Bonferroni-Holm* correction. Different letters indicate significant differences at $P < 0.05$

Chapter 4:

Kairomone-induced changes in foraging activity of the larval ectoparasitoid *Holepyris sylvanidis* are linked with an increased number of male parasitoid offspring.

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Kairomone-induced changes in foraging activity of the larval ectoparasitoid *Holepyris sylvanidis* are linked with an increased number of male parasitoid offspring

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Interactions between stored product pest insects and their parasitoids are well known to be mediated by infochemicals. However, little knowledge is available about the extent to which parasitoid responses to host kairomones improve host search and parasitization success. Here, we tested whether the release of host-specific kairomones of the confused flour beetle, *Tribolium confusum*, improves host finding of the larval ectoparasitoid *Holepyris sylvanidis*. Previous studies revealed that volatiles from host larval feces [i.e., (*E*)-2-nonenal and 1-pentadecene] attract the parasitoid. To determine the most attractive concentration of these two key components, we conduced Y-tube olfactometer bioassays. Most female parasitoids were attracted to a mixture of (*E*)-2-nonenal and 1-pentadecene at the lowest concentration, while the highest concentration was repellent. Volatiles from host adults (methyl-*p*-benzoquinone and 4,8-dimethyldecanal) did not attract the parasitoid females at any of the concentrations tested. In flight cage experiments, we analyzed the host finding success of *H. sylvanidis* by offering host larvae in a Petri dish for 3days (i) with additionally applied host larval kairomones in the most attractive concentration (test) or (ii) without additional kairomones (control). In test cages, the parasitoids removed a significantly higher number of paralyzed host larvae from the initial population within 48h than in control cages. After 72h, significantly more host larvae were parasitized in test cages than in the control. The offspring of parasitoids in test cages differed from the one in control cages by total numbers and especially the number of males. Significantly more parasitoids emerged from the parasitized host larvae in test cages, with enhanced male offspring. Our study demonstrates that the parasitoid's response to host-associated volatiles can improve parasitization success but also affects the number of males in the parasitoids' progeny.

KEYWORDS

Bethylidae, 1-pentadecene, *(E)*-2-nonenal, host finding, stored product protection, *Tribolium confusum*, Tenebrionidae

1. Introduction

Biological control measures against insect pests have become increasingly important in agriculture in recent decades as knowledge grows about the side effects of conventional chemical pesticides on human health and environment (Cox, 2004; van Lenteren, 2012; van Lenteren et al., 2018). Biologically based approaches such as the use of entomophagous insects (e.g., parasitic wasps) and the application of semiochemicals (e.g., sex pheromones of pest species for monitoring, mass trapping or mating disruption) are already implemented to some extent in integrated management of stored product pests. However, their potential is far from being fully exploited (Schöller et al., 1997; Schöller, 1998; Trematerra, 2012; Fürstenau and Kroos, 2020). For instance, nearly 500 parasitoid species are known to be natural enemies of various insects that attack stored plant products (Hagstrum and Subramanyam, 2009). But so far, only parasitoids belonging to the families Bethylidae and Pteromalidae are commercially available for biological control of stored product pest beetles (Flinn and Schöller, 2012; Amante et al., 2017b; van Lenteren et al., 2018). There are several reasons for the discrepancy between the wide variety of known parasitoids and the low number of commercially available ones. One reason are the costs and efforts that need to be invested for maintaining a rearing of parasitoids (van Lenteren and Tommasini, 1999; Parra and Coelho, 2022). Another one may be our limited knowledge about the biology and odor-mediated host-searching behavior of many species. Such knowledge is necessary for both efficient use of parasitoids in controlling a pest population and for a deeper understanding of the interaction of parasitoids and host insects via infochemicals (Fürstenau and Kroos, 2020).

Foraging parasitoids can eavesdrop on the intraspecific chemical communication of their host species and respond to the sex or aggregation pheromones for host location and recognition. In addition, other volatiles directly associated with the host (e.g., fecal volatiles or compounds on the host's cuticle) as well as indirectly associated ones (e.g., host feeding substrate or compounds belonging to different developmental stages of the host) also mediate host finding behavior in parasitoids (Vet and Dicke, 1992; Steidle and van Loon, 2003). The identification of host-specific kairomones is crucial for successful application of parasitoids in biological control of storage beetles. One of the major weaknesses of parasitoid use in stored product protection is the poor knowledge about these kairomones that could be used as lures in traps for monitoring parasitoid population densities in storage facilities (Schöller, 1998). Mass release of captured parasitoids could occur with pinpoint accuracy where needed (Trematerra, 2012). Furthermore, application of host-specific kairomones might modify the host-search of parasitoids (Cox, 2004).

Stored product parasitoids have the potential to locate their hosts up to 10 m away in empty storages and up to 4 m horizontally and vertically in grain commodities (Steidle and Schöller, 2002; Niedermayer et al., 2016). However, if local pest populations are widely dispersed, parasitoids might first examine a pest-free area and require more foraging time (Schöller, 1998). Release of synthetic host odors are known to attract parasitoids, guide them directly to an infestation site and increase the host searching activity (Cox, 2004; Phillips and Throne, 2010). The success of biological control of stored product insects by release of parasitoids might be improved by additional application of host kairomones if these host odors improve the parasitoid's host finding and parasitization success (Gross et al., 1984; Cox, 2004). Despite the potential of host-associated kairomones to enhance biological control of stored product pests, little is known about whether or how the additional release of host-specific kairomones influences host search and host-finding success of parasitoids.

We addressed this gap in knowledge by studying the behavioral responses of the larval ectoparasitoid *Holepyris sylvanidis* (Bréthes) (Hymenoptera: Bethylidae) to host kairomones. This parasitoid species feeds destructively and oviposits on larvae of several stored product pest beetles, in particular of the genus *Tribolium* (Evans, 1969; Amante et al., 2017a,b; Awater-Salendo et al., 2020). For oviposition, the female paralyzes a host larva and drags it to a sheltered site where the actual parasitization takes place and the parasitoid progeny can develop on its host (Ahmed et al., 1997). Two kairomonal key components, (*E*)-2-nonenal and 1-pentadecene released from larval feces of the confused flour beetle, *Tribolium confusum* (Du Val) (Coleoptera: Tenebrionidae) are known to attract *H. sylvanidis* over a short range (Fürstenau et al., 2016). While non-infested host feeding substrate (i.e., wheat grist) did not attract the parasitoid, infested wheat grist that naturally releases the kairomonal components, is highly attractive (Fürstenau et al., 2016). Other compounds involved in host location and host recognition of *H. sylvanidis* are long-chain cuticular hydrocarbons, in particular the methylated ones (Fürstenau and Hilker, 2017; Awater-Salendo et al., 2020, 2021).

A field study by Adler et al. (2012) on the biological control of *T. confusum* showed that a local pest population in a flour mill was successfully controlled by a biweekly mass release of *H. sylvanidis* over a period of 6 months. However, because of the lack of suitable traps, it was not possible to monitor released parasitoids during the experiment and to determine their whereabouts at the end. Based on the results from our previous laboratory tests, (*E*)-2-nonenal and 1-pentadecene represent potential candidate components for such monitoring traps, but this has not been tested so far. Moreover, it is still unclear whether *H. sylvanidis* also uses other odor cues associated with different developmental stages of *T. confusum* than the larval stage. Cues from other developmental stages of the host species than the larvae might provide reliable information about the future presence of host larvae. Therefore, in the present study we tested whether two compounds identified from volatiles of adults of different *Tribolium* species (Suzuki and Sugawara, 1979; Suzuki, 1980; Faustini and Burkholder, 1987) can attract *H. sylvanidis* females. These two compounds are methyl-*p*-benzoquinone (MBQ) and 4,8-dimethyldecanal (DMD). MBQ is released with the defensive secretion of adult *Tribolium*, whereas DMD has been described as part of the aggregation pheromone and is already used for monitoring *Tribolium* species (Arnaud et al., 2002; Verheggen et al., 2007; Duehl et al., 2011; McKay et al., 2019).

In detail, we studied the following questions: (i) Which concentration of a mix of the two key larval kairomonal compounds, i.e., (*E*)-2-nonenal and 1-pentadecene (2CM) needs to be added to non-infested host feeding substrate (wheat grist) to render it as attractive as infested feeding substrate? (ii) Is *H. sylvanidis* also attracted to volatile compounds associated with other developmental

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Abbreviations: 2CM, Two-Component-Mix containing (*E*)-2-nonenal and 1-pentadecene; DMD, 4,8-dimethyldecanal; IG, infested wheat grist; MBQ, methyl-*p*-benzoquinone; NIG, non-infested wheat grist.

stages than beetle larvae (MBQ and DMD) for host search? (iii) Does the release of the specifically host-associated volatiles (2CM) enhance the host search activity and host-finding success of *H. sylvanidis*?

Therefore, we first performed Y-tube-olfactometer bioassays with different concentrations of 2CM, MBQ and DMD to select the concentration of those compounds which were the most attractive for parasitoid females. Then, we conducted flight cage experiments to analyze how these host larval kairomones affect the host finding behavior of *H. sylvanidis*. In these cages, we offered 20 host larvae for 3 days with additionally applied host larval kairomones (test) or without (control) and determined host search activity and efficacy, parasitization rate as well as the sex ratio of parasitoid progeny.

2. Methods and material

2.1. Insects

Holepyris sylvanidis and *T. confusum* specimens were reared at the Julius Kühn Institute (Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection Berlin, Germany) as previously described by Fürstenau et al. (2016). For all bioassays, one- to five-day old *H. sylvanidis* females without prior oviposition experience and 4th instar *T. confusum* larvae were used.

2.2. Preparation of kairomonal stimuli

The two-component mix (2CM) of host larval volatiles comprised 1-pentadecene (95%, TCI Europe, Eschborn, Germany) and (*E*)-2-nonenal (97%, Sigma Aldrich, Taufkirchen, Germany) in a 1:1 ratio. The following concentrations were prepared: 0.02, 0.2, and 2 μg μL^{-1} in *n*-hexane (>98%, VWR, Radnor, United States).

To test the parasitoid's behavioral response to volatiles releases from *Tribolium* adults, MBQ (98% Sigma Aldrich, Taufkirchen, Germany) was diluted in methanol (99.8%, Merck, Darmstadt, Germany) at the following concentrations: 0.1, 1, 10, and 100 ng μL^{-1}. All solutions were stored at −20°C until usage. Since DMD was not commercially available, we purchased pheromone dispensers with DMD from Trécé Incorporated (Adair, United States). These pheromones dispensers were stored at 5 ± 1°C until usage. Before the DMD-dispensers were used in olfactometer bioassays, they had been placed in a fuming hood for 24 h.

2.3. Y-tube olfactometer bioassays

Behavioral responses of *H. sylvanidis* females to directly and indirectly host-associated volatiles were analyzed in a Y-shaped glass olfactometer. The olfactometer consisted of a main tube (21 cm long × 15 mm ID) with two 15 cm long arms at an angle of approximately 135° to each other. Each arm was connected via polytetrafluoroethylene (PTFE) tubing to a glass flask (50 mL). The end of each lateral arm was covered with fine-meshed gauze to prevent parasitoid females from escaping. A lid with two entrances sealed each glass flask which contained a test odor sample at the test side and a control sample at the other side. Charcoal-filtered air passed the glass flasks with the samples, entered the Y-tube arms at 100 mL min^{-1} and flowed to the entrance of the main tube where a parasitoid female was released to the olfactometer. The system was horizontally placed onto a table and homogeneously illuminated by a dimmed neon office lamp (30 lx) placed 50 cm above the olfactometer. All bioassays were conducted under similar conditions from 9:00–17:00 h at 25 ± 1°C and 38 ± 0.2% RH.

Prior to the start of bioassays, Y-tube olfactometers, flasks, and connection tubes were cleaned with a detergent, followed by rinsing with distilled water and 70% ethanol solution (>96%, Berkel AHK, Ludwigshafen, Germany) before being dried in an oven for 3.5 h at 175°C. Unmated parasitoid females were removed from the rearing and kept in plastic Petri dishes (5.0 cm diam., five individuals in each Petri dish) in the experimental room to acclimatize for 1 h before they were placed at the base of the main arm of the olfactometer.

We determined which concentration of kairomones needs to be added to non-infested wheat grist to reach the attractiveness of wheat grist infested with *T. confusum* larvae. Therefore, we first run bioassays with non-infested wheat grist (NIG) versus air and then infested wheat grist (IG) versus air (Table 1, #1, 2, 7, 12) to check whether our previous results (Fürstenau et al., 2016), which were obtained by using a 4-field olfactometer, can be confirmed with the set-up applied here. We used 5 g finely ground wheat grist (*Triticum aestivum* Linnaeus 1753). For the IG, we took 5 g infested wheat grist from rearing jars (450 mL) in which about 1,000 *T. confusum* larvae developed from hatching on for 5 weeks and were removed from the grist prior to the bioassay (compare Fürstenau et al., 2016).

We tested the parasitoid's behavioral response to (i) directly host-associated odors from *T. confusum* larval feces (2CM) and (ii) indirectly host-associated odors from *Tribolium* adult volatiles (MBQ and DMD). To test different concentrations of 2CM (Table 1, #3–6), 10 μL of the respective kairomone (test) and 10 μL of the solvent *n*-hexane (control) were applied on Whatman filter papers (55 mm diam.) and could evaporate for 5 min. The loaded filter papers were then placed into the glass flasks. Likewise, different concentrations of MBQ were tested, but methanol was used for control (Table 1, #8–11). To compare the attractiveness of infested grist with the one of non-infested grist augmented with DMD, we placed a DMD dispenser (compare 2.2) together with 5 g NIG into the test glass flask and 5 g NIG into the control flask (Table 1, #13).

In the beginning of each bioassay, one *H. sylvanidis* female was released at the bottom of the main arm that was immediately closed with a fine-meshed gauze. The behavioral response of each test individual was observed for max. 300 s. When an upwind-walking individual reached the middle of one arm (crossing a line drawn 30 mm beyond the intersection) and did not return to the intersection within 10 s, this was recorded as positive response to the odor offered in the respective olfactometer arm. Thereafter, the experiment was stopped, and the test individual was removed. After five tests, the Y-olfactometer and glass flask with samples were replaced by new ones. In addition, positions of test and control arms were changed to avoid biased results due to possible side preference of test individuals. A preliminary test in the Y-tube olfactometer with no odor (air vs. air) showed that the parasitoid females showed no side preferences, indicating no positional effect of the experimental setup. Parasitoid females, which had rested for more than 50% of observation time and did not show any preference, were replaced by new ones and were not included in the statistical analysis. This was the case in less than 1% (two observations) of all bioassays listed in Table 1.

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TABLE 1 Overview of Y-tube olfactometer bioassays to analyze the attraction of ***Holepyris sylvanidis*** females to directly and indirectly host-associated volatiles.

Bioassay	#	Comparison[1]	*N*[2]
(A) Volatiles associated with *T. confusum* larvae	1	NIG[3] vs. air	30
	2	IG[4] vs. air	30
	3	$2CM_{low}$[5] vs. *n*-hexane[8]	30
	4	NIG[3] + $2CM_{low}$[5] vs. *n*-hexane[8] + NIG[3]	30
	5	NIG[3] + $2CM_{med}$[6] vs. *n*-hexane[8] + NIG[3]	30
	6	NIG[3] + $2CM_{high}$[7] vs. *n*-hexane[8] + NIG[3]	30
(B) Volatiles associated with *T. confusum* adults	7	IG[4] vs. air	30
	8	NIG[3] + MBQ_{low}[9] vs. methanol[13] + NIG[3]	30
	9	NIG[3] + MBQ_{med}[10] vs. methanol[13] + NIG[3]	30
	10	NIG[3] + MBQ_{high}[11] vs. methanol[13] + NIG[3]	30
	11	NIG[3] + $MBQ_{veryhigh}$[12] vs. methanol[13] + NIG[3]	30
	12	IG[4] vs. air	20
	13	DMD[14] + NIG[3] vs. NIG[3]	20

[1]For the preparation of single stimuli see experimental part.
[2]Number of replicates.
[3]NIG = 1 g non-infested wheat grist.
[4]IG = 0.5g infested wheat grist.
2CM = Two-component mix [(*E*)-2-nonenal + 1-pentadecene, 1:1 in *n*-hexane] in three different concentrations (10 µL each applied):
[5]= 0.02 µg μL^{-1}, in total 0.2 µg.
[6]= 0.2 µg μL^{-1}, in total 2 µg.
[7]= 2 µg μL^{-1}, in total 20 µg.
[8]= 10 µL *n*-hexane.
MBQ = Methyl-*p*-benzoquinone (in methanol) in four different concentrations (10 µL each applied):
[9]= 0.1 ng μL^{-1}, in total 1 ng.
[10]= 1 ng μL^{-1}, in total 10 ng.
[11]= 10 ng μL^{-1}, in total 100 ng.
[12]= 100 ng μL^{-1}, in total 1,000 ng.
[13]= 10 µL methanol.
[14]DMD = Pheromone dispenser with 4,8-dimethyl decanal purchased by Trécé Incorporated (unknown concentration).

Thirty parasitoid females were tested in bioassays offering different concentrations of 2CM or MBQ ($N = 30$ per compound and concentration). When the effect of DMD on the parasitoid's olfactory response was studied, twenty replicates were conducted ($N = 20$).

2.4. Behavioral bioassays in flight cages

To study possible effects of host-associated volatiles (= 2CM in the lowest concentration = $2CM_{low}$) on the host search and host-finding of *H. sylvanidis* females, we performed behavioral tests using flight cages (32.5 × 32.5 × 77.0 cm, 150 × 150 mesh, cage with sleeve opening, BugDorm, Taiwan). The experiment was conducted in two identical rooms (3 × 4 × 2.5 m), a test and a control room to avoid any biased effects (e.g., by diffusing volatiles from 2CM replicates over control replicates). Both rooms were not air-conditioned or heated. In each room, six flight cages were placed. In the test room, parasitoids were offered host larvae that fed upon wheat grist in the presence of 2CM additionally applied to dispensers (test treatment). In the control room, no 2CM but only hexane was applied in addition to host larvae and wheat grist (control treatment). In both the test and control room, flight cages were placed in two rows. In each row, three cages were located right next to each other, while the distance between the two rows was approx. 63 cm. In front of each row an oscillating table fan (14.4 × 8.0 × 18.8 cm, 5,000 mAh, Comlife, China) was placed behind the side of the cage where the host odor source was. The fan provided a smooth airflow from the site, where host larvae (and kairomonal host odors) were located, to the release points of the parasitoids (Supplementary Figure S1A). All replicates (= flight cages) in one room were treated equally. Two data loggers recorded room temperature and relative humidity in each room (on average 22 ± 1°C and 33 ± 7% RH). The experimental setup is outlined in Supplementary Figure S1A.

Twenty-four hours before starting the experiment, one- to five-day old *H. sylvanidis* males and females were taken from the permanent rearing and could mate in groups (three females and one male) for approximately 18 h.

On the day of the experiment, *H. sylvanidis* females were separated from males in the morning and brought into the experimental rooms for 2 h to adapt to local conditions. On one side of the flight cage 20 *T. confusum* larvae and *ca.* 10 g of finely grounded wheat grist were evenly distributed in an open Petri dish (55 mm diam) close to an attached rubber septum. Since *H. sylvanidis* females drag paralyzed host larvae to a shelter for parasitization (Ahmed et al., 1997), pipette tips (0.1–20 µL, Carl Roth, Karlsruhe, Germany) were evenly distributed in the cage as shelter sites (Supplementary Figure S1B). In the test treatment, we applied 10 µL $2CM_{low}$ (concentration 0.02 µg μL^{-1}, in total 0.2 µg) to the rubber septum and let the solvent evaporate for 5 min. In the control treatment, 10 µL *n*-hexane was used.

To start the experiment, three mated parasitoid females ($N = 3$ per cage) were released 70 cm away from the odor source on the other side of the flight cage (Supplementary Figure S1B).

The experiment took 72 h. Thereafter, we removed the parasitoid females, non-parasitized *T. confusum* larvae, pipette tips, the dispensers attached to lower parts of Petri dishes, and wheat grist from the cages. All cages were cleaned with 70% ethanol. In total, we repeated the experiments 10 times ($N = 6$ cages × 10 replicates each per control and test).

To analyze how host-associated volatiles (2CM) affect the parasitoid's host search and parasitization success, we determined the following parameters: host search activity efficacy in host search, parasitization rate of host larvae, and production of a new parasitoid generation (adult parasitoid offspring).

2.4.1. Host search activity

The number of host larvae dislocated by the parasitoids from the open Petri dish to the pipette tips or somewhere else in the flight cage, was considered a parameter of host search activity. We counted the number of dislocated host larvae at different time point (1, 24, 48, and 72 h after parasitoid release) without removing them from the cage. At the end of the experiment (after 72 h), however, the number of the counted larva did not always match the number of host larvae missing in the open Petri dish (=23 times during the flight cage experiment). This was probably due to the escape of some parasitoid females from the cage through tiny openings; indeed, sometimes, a parasitoid female was seen outside the cage. Therefore, in case of a mismatch between the number of found, dislocated host larvae and number of host larvae missing in the open Petri dish, we considered the number

of missing larvae as dislocated ones because host larvae could not leave the Petri dish on their own. We determined the mean number of these dislocated larvae at each time point in test and control cages. The overall dislocation rate was calculated as percentage of dislocated larvae of all offered larvae at the experiment's end (72h). In some cases, we could recover all offered *T. confusum* in the Petri dish 72h after bioassay start, indicating that no dislocation took place probably due to escaped parasitoids. Those replicates were excluded from further statistical analysis. This was the case for 13 test cages and 20 control cages.

2.4.2. Host search efficacy

For evaluation of the parasitoid's efficacy in host search, the number of host larvae with feeding marks inflicted by the parasitoids (e.g., brownish bite marks at the forelegs) and that of host larvae that were found dislocated, but without feeding marks were used. The efficacy in search for host larvae was calculated by determining the sum of these larvae as percent of the number of offered host larvae at the beginning of the experiment ($N=20$ host larvae per cage). Host larvae with feeding marks are not used for parasitization (Abdella et al., 1985).

2.4.3. Parasitization rate of *T. confusum* larvae

We determined the number of parasitized host larvae at the end of the experiment (72h). Parasitized host larvae were paralyzed, and one parasitoid egg was placed externally between the first hind pair of legs (approximately between the first and second abdominal sternite; Ahmed et al., 1997; Amante et al., 2017a). The parasitization rate was calculated as percentage parasitized larvae of the number of initially offered host larvae ($N=20$ per cage).

2.4.4. Parasitoid offspring

At the end of the experiment, parasitized host larvae were individually placed in Petri dishes (55mm diam.) and stored at 25±1°C, 65±5% RH and permanent darkness for further development. Four to 6weeks after egg deposition, *H. sylvanidis* adult offspring emerged from the host larvae. For 6weeks, we determined the number of adult female and male parasitoid individuals emerging from parasitized host larvae. For the mean number of all emerged *H. sylvanidis* offspring individuals and the mean overall emergence rate, we considered all emerged offspring including those of not identifiable sex. The emergence of the latter was indicated by empty parasitoid cocoons, but the sex could not be identified due to the disappearance of adults after emergence. The mean overall emergence rate was calculated by determining the total number of emerged adult parasitoid offspring as percent of the number of parasitized host larvae. In the case that a parasitoid larva died during its development and no parasitoid adult emerged during the observed period from a host larva, these host larvae were not included in further statistical analysis.

2.5. Statistics

All statistical analysis were performed in "R," version 4.1.3 (R Core Team, 2021).

For the evaluation of data obtained by the Y-tube olfactometer assays, we used a one-sided binomial test to analyze differences in the parasitoid's preference for simultaneously offered control and test stimuli. The null hypothesis was that percentages of test individuals choosing the test or the control stimulus were equal. Furthermore, we compared the parasitoid preference for IG and NIG plus kairomones by performing the test for equality of proportions (Newcombe, 1998a,b).

The behavioral parameters recorded in the flight cage experiment were first analyzed for normal distribution by the *Shapiro–Wilk* test. In the case of normal distribution, the behavioral parameters of parasitoids in test and control were compared by a Student's *t*-test. If data were not normally distributed, we applied the *Wilcoxon* rank-sum test for independent samples instead. To analyze if there were differences in the sex ratios of emerged parasitoid offspring that emerged in the test and control cages, we applied χ^2 goodness of fit test.

3. Results

3.1. Response of *Holepyris sylvanidis* to host-associated volatiles from *Tribolium confusum* larvae and adults

Adult parasitoid females strongly preferred infested wheat grist (IG, Figure 1). Almost 90% of the parasitoids chose the test arm provided with IG odor over the control arm, whereas they did not prefer non-infested wheat grist (NIG, 57%).

The response of parasitoids to directly host-associated kairomonal odor (2CM) offered on filter paper in the olfactometer assay were as follows (Figure 1): A low concentration ($2CM_{low}$) was significantly preferred by 77% of *H. sylvanidis* females when tested against the control odor. In comparison to the parasitoid's response to IG, fewer test individuals preferred $2CM_{low}$ but there was no significant difference. When offering a combination of $2CM_{low}$ and NIG, the number of test individuals preferred this odor increased (87%); this number was as about high as that preferred IG odor. However, the higher the concentration of (*E*)-2-nonenal and 1-pentadecene was in the 2CM odor, the fewer individuals preferred the test arm. Only 47% of test individuals preferred the odor of the intermediate 2CM concentration ($2CM_{med}$) over the control. In bioassays with the highest concentration of 2CM ($2CM_{high}$) 66% of all test individuals were repelled. The number of parasitoids, which preferred the odor of $2CM_{med}$ and $2CM_{high}$, was significantly lower than the number of parasitoids, which preferred IG odor. Thus, regardless of the presence or absence of non-infested wheat grist, only a low concentration of 2CM turned out to be as attractive to female *H. sylvanidis* as odor of infested grist.

The indirectly host-associated volatiles from *T. confusum* adults elicited the following responses of the parasitoids (Figure 2): Odor of all four tested concentrations of MBQ (10µL each applied onto filter paper) was not significantly attractive when tested against NIG combined with the solvent methanol (Figure 2A). The highest MBQ concentration ($MBW_{very\ high}$, 100ngµL^{-1}, in total 1,000ng) even had a significant repellent effect on the parasitoids. The percentage of parasitoids that chose the olfactometer arm provided with MBQ odor decreased from about 40% when testing the lowest MBQ concentration (MBW_{low}, 0.1ngµL^{-1}, in total 1ng) or high MBQ concentration (MBQ_{high}, 10ngµL^{-1}, in total 100ng) to about 17%

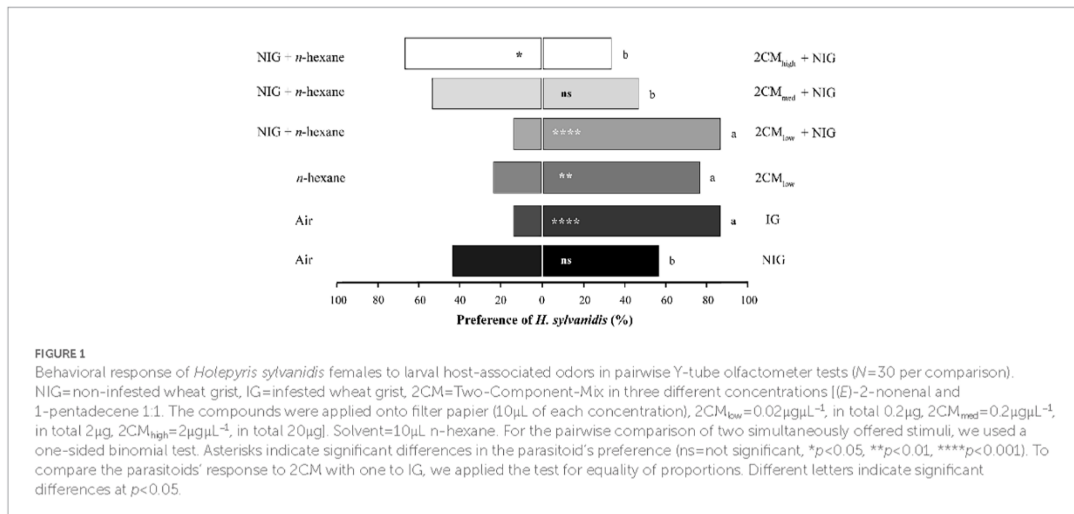

FIGURE 1
Behavioral response of *Holepyris sylvanidis* females to larval host-associated odors in pairwise Y-tube olfactometer tests (N=30 per comparison). NIG=non-infested wheat grist, IG=infested wheat grist, 2CM=Two-Component-Mix in three different concentrations [(E)-2-nonenal and 1-pentadecene 1:1. The compounds were applied onto filter papier (10µL of each concentration), $2CM_{low}$=0.02µgµL^{-1}, in total 0.2µg, $2CM_{med}$=0.2µgµL^{-1}, in total 2µg, $2CM_{high}$=2µgµL^{-1}, in total 20µg]. Solvent=10µL n-hexane. For the pairwise comparison of two simultaneously offered stimuli, we used a one-sided binomial test. Asterisks indicate significant differences in the parasitoid's preference (ns=not significant, *p<0.05, **p<0.01, ****p<0.001). To compare the parasitoids' response to 2CM with one to IG, we applied the test for equality of proportions. Different letters indicate significant differences at p<0.05.

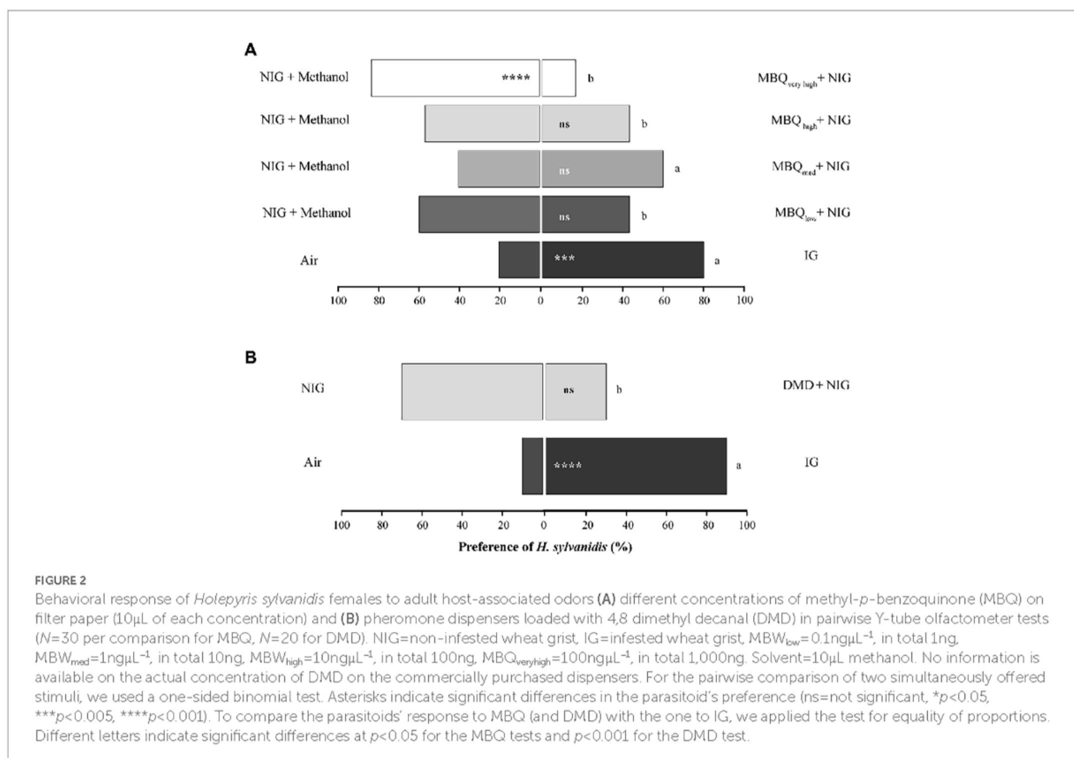

FIGURE 2
Behavioral response of *Holepyris sylvanidis* females to adult host-associated odors **(A)** different concentrations of methyl-p-benzoquinone (MBQ) on filter paper (10µL of each concentration) and **(B)** pheromone dispensers loaded with 4,8 dimethyl decanal (DMD) in pairwise Y-tube olfactometer tests (N=30 per comparison for MBQ, N=20 for DMD). NIG=non-infested wheat grist, IG=infested wheat grist, MBW_{low}=0.1ngµL^{-1}, in total 1ng, MBW_{med}=1ngµL^{-1}, in total 10ng, MBW_{high}=10ngµL^{-1}, in total 100ng, $MBQ_{veryhigh}$=100ngµL^{-1}, in total 1,000ng. Solvent=10µL methanol. No information is available on the actual concentration of DMD on the commercially purchased dispensers. For the pairwise comparison of two simultaneously offered stimuli, we used a one-sided binomial test. Asterisks indicate significant differences in the parasitoid's preference (ns=not significant, *p<0.05, ***p<0.005, ****p<0.001). To compare the parasitoids' response to MBQ (and DMD) with the one to IG, we applied the test for equality of proportions. Different letters indicate significant differences at p<0.05 for the MBQ tests and p<0.001 for the DMD test.

when testing the highest (repellent) MBQ concentration ($MBW_{very\,high}$). This trend of a decreasing number of parasitoids choosing the MBQ-provided olfactometer arm when the MBQ concentration increased was interrupted by the response of parasitoids to the intermediate MBQ concentration (MBW_{med}, 1.0 ng µL^{-1}, in total 10 ng). About 60% of the tested individuals chose the olfactometer arm with MBQ_{med}. While this proportion of MBQ-responsive females did not significantly differ from the one responding to NIG only, it was neither statistically different from the percentage of females (~ 80%) responding to the attractive odor of IG. The percentage of parasitoids

responding positively to the other tested MBQ concentrations was always significantly lower than the one responding positively to IG odor (Figure 2A). Thus, the behavioral response of the parasitoids to MBQ was dependent on the MBQ concentration.

When odor of DMD released from a rubber septum was combined with NIG, it did not elicit a significant behavioral response of the parasitoids. Most test individuals (70%) chose the control arm (with NIG only), while 30% went into the test arm supplied with DMD plus NIG, this difference was not statistically significant. The percentage of test individuals choosing the test arm was significantly lower than the one attracted by IG odor (90%, Figure 2B). All details about the statistical evaluation on the above-mentioned results are given in Supplementary Table S1.

3.2. The influence of 2CM on host search of *H. sylvanidis*

The directly host-associated kairomones (2CM, 10 μL applied onto a rubber septum) exerted significant effects on various parameters of the host location behavior of *H. sylvanidis* in a flight cage.

3.2.1. Host search activity

When taking the number of dislocated host larvae as indicator for host search activity, the offered low concentration of 2CM ($2CM_{low}$, 0.02 μg μL^{-1}, in total 0.2 μg) induced the search activity during the first 2 days after experiment start (Figure 3). Already 1 h after parasitoid release, parasitoid females had dislocated *T. confusum* larvae in test cages where 2CM was additionally applied. The number of dislocated *T. confusum* larvae was significantly higher in the test cages than in control cages where no host larvae were dislocated at this time point. Twenty-four hours after parasitoid release dislocated host larvae were found in both test and control cages but their number was significantly higher in the test cages than in the control ones. After 48 h the number of dislocated host larvae duplicated both in test and control flight cages. Nevertheless, parasitoid females dislocated significantly more host larvae in the presence of 2CM than in the control. While the number of dislocated host larvae was still higher in the test cages than in the controls at the end of the experiment (72 h), this difference was not significant anymore when compared to the control cages (Figure 3). Overall, about one third of the offered *T. confusum* larvae were dislocated in cages provided with 2CM while it was only about 25% in the control cages (Table 2). Thus, the 2CM treatment enhanced the search activity of the parasitoids for about 2 days after application of the kairomones, but not for longer.

3.2.2. Host search efficacy

At the end of experiments, we detected beetle larvae with host feeding marks in all treatments, but their number did not significantly differ between test and control treatment (Table 2). Nevertheless, we used the number of larvae with host feeding marks and added them to the total number of dislocated *T. confusum* larvae found without feeding marks within 72 h for calculating the efficacy of *H. sylvanidis* females as biological control agent. In the presence of 2CM the parasitoid's search efficacy was reflected by 32.55% of all host

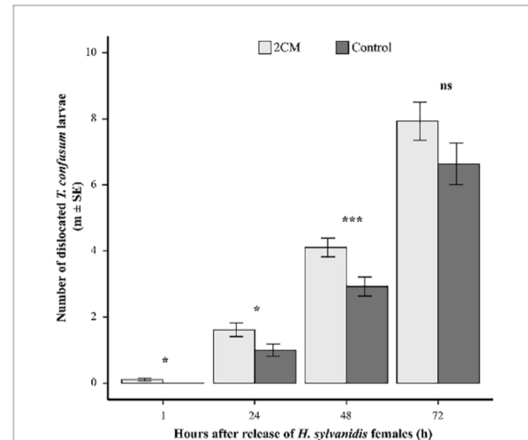

FIGURE 3
Mean number of dislocated *Tribolium confusum* larvae in a flight cage trial in the presence or absence of additionally applied larval host kairomones [2CM=(*E*)-2-nonenal and 1-pentadecene, ratio 1:1 in *n*-hexane; 10μL applied onto a rubber septum, concentration=0.02μgμL^{-1}, in total 0.2μg]. Dislocated larvae were counted after 1, 24, 48, and 72h (*N*=40–47 per treatment). For each time interval, differences between the treatments were analyzed by applying a *student t*-test or *Wilcoxon* rank-sum test for independent data (ns=not significant, *p>0.05, ***p>0.005).

larvae offered that were dislocated and/or fed upon. This percentage is significantly higher than that in the absence of host-associated volatiles (~25%), indicating that the host search efficacy is inducible by 2CM.

3.2.3. Parasitization rate

When counting the number of visibly parasitized host larvae, *H. sylvanidis* parasitized in average significantly more *T. confusum* larvae per cage in the presence of 2CM when compared to the control (Table 2). When calculating the parasitization rate per 20 host larvae offered in the beginning of the experiment (*N* = 20) per cage, the parasitization rate was significantly higher in the test treatment than in the control.

3.2.4. Parasitoid offspring

Four-to-six weeks after the bioassays, we recorded the number of adult parasitoids that successfully developed in visibly parasitized larvae and emerged from them. When taking all visibly parasitized larvae as 100%, about three quarters of them in the 2CM-treated cages and nearly 60% of them in the control cages delivered adult parasitoids (Table 2). While the difference in this mean overall emergence rate between test and control cages was not significant, the mean number of all emerged *H. sylvanidis* offspring individuals was significantly enhanced in the test cages treated with 2CM.

Regarding the sex of the parasitoid offspring, significantly more male offspring emerged from parasitized host larvae that were in the 2CM-treated test cages compared to that from parasitized host larvae in the control cages (Table 2). In contrast, there was no significant difference in the number of female offspring emerging from parasitized larvae in test and control cages. The ratio of male to female offspring was about 1:2 in the test cages and about 1:3 in the control

TABLE 2 Effect of a mixture of synthetic host larval volatiles (2CM) on the host finding success of ***Holepyris sylvanidis*** females determined by host search activity and efficacy, parasitization and offspring emergence.

Evaluation parameter		Treatments[1]	
		$2CM_{low}$ Mean ± SE[2]	Control Mean ± SE[2]
(1) Host search activity and efficacy	Total mean number of dislocated *T. confusum* larvae[3]	7.93 ± 0.58^a	6.63 ± 0.63^a
	Dislocation rate[3] (%)	30.85 ± 2.42^a	24.13 ± 2.53^a
	Mean number of *T. confusum* larvae with host feeding marks	0.30 ± 0.09^a	0.20 ± 0.09^a
	Efficacy of *H. sylvanidis*[4] (%)	32.55 ± 2.59^a	25.38 ± 2.68^b
(2) Parasitization	Mean number of parasitized *T. confusum* larvae	3.74 ± 0.34^a	2.63 ± 0.39^b
	Parasitization rate[5] (%)	18.72 ± 1.70^a	13.13 ± 1.93^b
(3) Offspring emergence	Mean number of emerged *H. sylvanidis* females	2.00 ± 0.27^a	1.56 ± 0.30^a
	Mean number of emerged *H. sylvanidis* males	1.16 ± 0.21^a	0.56 ± 0.12^b
	Mean number of all emerged *H. sylvanidis* offspring individuals[6]	3.15 ± 0.34^a	2.29 ± 0.37^b
	Mean overall emergence rate[7] (%)	73.37 ± 5.28^a	58.56 ± 6.41^a
	Sex ratio (male: females)[8]	$1: 1.73^a$	$1: 2.80^a$

[1] $2CM_{low} = 0.02\ \mu g\ \mu L^{-1}$, 10 µL applied, in total 0.2 µg (*E*)-2-nonenal and 1-pentadecene (1:1) control = *n*-hexane.
[2] Mean numbers were calculated per cage. Different letters indicate significant differences between test and control treatment (*Wilcoxon* rank-sum test, $p < 0.05$).
[3] Calculated on the number of dislocated *T. confusum* larvae.
[4] Calculated on the number of dislocated *T. confusum* larvae and those larvae with host feeding marks.
[5] Calculated on the number of offered *T. confusum* larvae ($N = 20$ per cage).
[6] Male and female offspring as well as those emerged *H. sylvanidis* offspring whose sex could not be identified.
[7] Calculated on the total number of emerged *H. sylvanidis* offspring divided by the number of parasitized *T. confusum* larvae.
[8] Differences in the sex ratio was analyzed by χ^2 goodness of fit test.

cages but this shift in ratio was not significant. Thus, the additional application of 2CM changed the total parasitoid offspring production, particularly due to the increase of male progeny.

An overview of detailed statistics on the above-mentioned parameters is given in Supplementary Table S2.

4. Discussion

Our study showed that larval host kairomones can significantly enhance the host search activity, efficacy and parasitization rate of the larval ectoparasitoid *H. sylvanidis*. In contrast, compounds released by adult hosts for aggregation and defense did not elicit positive responses by the parasitoids. The parasitoid's responses to the larval host kairomonal compounds (2CM) as well as to the compounds released by adult hosts (MBQ, DMD) were concentration dependent. The high concentrations repelled the parasitoids. Interestingly, parasitoid offspring produced in the presence of applied larval host kairomones was increased, more male offspring emerged, and the sex ratio tended to shift toward males ($p = 0.13$).

High concentrations of larval host kairomones indicate a high host density and thus, a rich resource for the parasitoids. So why do high concentrations of these compounds repel the parasitoids? The attractive concentration of 2CM tested here ($2CM_{low}$, $0.02\ \mu g\ \mu L^{-1}$; 10 µL applied) is equivalent to the quantities of larval feces released by more than 100,000 *T. confusum* host larvae in the 4th instar (calculated based on data by Fürstenau et al., 2016). Thus, even the lowest concentration of 2CM tested here already indicates a very high host population density. Higher concentrations of 2CM indicate enormously high host densities. Such extraordinarily high host densities might be linked with reduced host quality due to high intraspecific competition among the hosts and a strong risk of entomopathogen infection; it will be beneficial for *H. sylvanidis* to avoid risky, low-quality hosts in which the chance to successfully produce healthy offspring would be low.

Like for the larval host kairomones, one of the tested indirectly host-associated volatiles, i.e., MBQ, also elicited a repellent effect at a very high concentration. A very high concentration of this compound ($100\ ng\ \mu L^{-1}$; 10 µL applied), which is released with the defensive secretion of adults (von Endt and Wheeler, 1971; Wirtz et al., 1978), might indicate a high risk of predation by entomophagous enemies. The parasitoids will benefit from avoiding such habitats. In contrast, the lower concentrations of MBQ tested here ($0.1, 1, 10\ ng\ \mu L^{-1}$; 10 µL applied) did not elicit any significant response of the parasitoids, showing quite a "relaxed" behavior in the presence of this aggressive odor. The tested DMD odor was released from purchased dispensers with this aggregation pheromone compound, but the exact concentration is unknown. Females of *H. sylvanidis* showed no significant preference for or avoidance of this odor. So far, DMD was only detected in odor released by adult host males (Arnaud et al., 2002). If DMD would also be released by adult host females, the odor might indicate a likelihood of host eggs to be laid, from which host larvae will hatch. Thus, the lack of a significant response of the parasitoids to DMD might be explained by the fact that DMD is released only by host males, and thus does probably not provide reliable indication for the presence of host larvae or of host eggs to be laid by host females.

Our results suggest at a first glance that the kairomone function of host odor is specific for the host's developmental stage in the interaction between *T. confusum* and *H. sylvanidis*, since attraction was only found for 1-pentadecene and (*E*)-2-nonenal, introduced here and known as typical host larval kairomones. However, 1-pentadecene has also been detected in odor released from adult male and female *Tribolium* species (von Endt and Wheeler, 1971; Keville and

Kannowski, 1975; Arnaud et al., 2002; Verheggen et al., 2007). Thus, attraction of parasitoids is obviously possible by odor components that larval and adult hosts have in common.

The above-discussed results were obtained by olfactometer assays. The flight cage bioassays revealed that the host larval kairomones (2CM) can also significantly affect the parasitoid's behavioral response under conditions mimicking the natural ones a bit more than the olfactometer bioassays. The tiny parasitoids were shown to successfully and quickly locate hosts (and host odor) that is 70 cm away from them.

The loss of the positive effect of 2CM on host search activity 72 h after the start of the flight cage bioassays is probably due to the evaporation of these kairomones. An analysis of the release rate of 2CM from a rubber septum (as applied in the flight cage bioassays) revealed a very rapid release of the highly volatile (*E*)-2-nonenal within 2 days, while 1-pentadecene was released continuously only in slow quantities (Supplementary material: Release rate; Supplementary Figure S2). The co-incidence of the loss of effect of 2CM on the parasitoids' search activity after 2 days (Figure 3) and the high evaporation of (*E*)-2-nonenal within 2 days suggests that the significant behavioral effects of 2CM on host search activity is especially induced by (*E*)-2-nonenal. On the other hand, our previous bioassays (in a 4-field olfactometer) showed no difference in the attractiveness of (*E*)-2-nonenal and 1-pentadecene.

At the beginning of the experiment, we observed that *H. sylvanidis* females in the test cages immediately walked toward the odor source (2CM dispensers) after release, while parasitoid females in the control cages remained at the release site. But during the experiment, these females were observed dispersing throughout the control cages. One further explanation for the loss of the effect of 2CM on the search activity of *H. sylvanidis* 72 h after experiment start could be also that the less readily and actively searching parasitoids in the control cages could finally catch up with those in the test cages in terms of the number of dislocated larvae because they had that much time for searching (72 h). It is very likely that parasitoids in the control also have completely searched the small cages within 3 days. As a result, the number of dislocated host larvae between test and control treatment converged at the end of the experiment.

The enhancer effect of 2CM on the parasitization rate of *H. sylvanidis* (calculated based on the number of offered *T. confusum* larvae) might be a consequence of the increased search activity of the parasitoids during the first 2 days of the experiment. Our results are consistent with those of Gross et al. (1984), who showed that the additional application of kairomones (here: hexane extracts of corn earworm scales, *Heliothis zea*) can enhance the parasitization activity of the egg parasitoid *Trichogramma pretiosum*. We also suppose that the parasitization rates in both the test and control treatments were likely higher than our results indicate. In some cases, at the end of experiment we could not recover all of the 20 offered *T. confusum* larvae. This was probably because some parasitoid females found a way out the cage and were able to drag paralyzed host larvae outside the cage. We do not know exactly whether *H. sylvanidis* used these dislocated host larvae for oviposition. However, when calculating the parasitization rate based on the number of parasitized, dislocated larvae found (all found dislocated larvae = 100%), the parasitization rate was about 65% in the test cages and about 50% in the controls. This suggests a higher parasitization rate in both treatments. Further studies on a larger scale (e.g., as reported in Niedermayer et al., 2016) are necessary to evaluate the potential of host-associated odors (2CM) on long-range host finding and parasitization rate of *H. sylvanidis*. However, it should be considered that the application of synthetic kairomones in stored product environments might disturb the host search of parasitoids if these compounds interfere with the kairomones naturally emitted by the host, thus preventing the parasitoids from detecting the exact location of their hosts. Therefore, the concentration of synthetic kairomones should be chosen very carefully.

The application of 2CM resulted in an increased number of male offspring individuals, which contributed to an overall increase in the total number of emerged offspring individuals in test cages. *Holepyris sylvanidis* is an arrhenotocous species. Male offspring hatches from unfertilized eggs and female offspring from fertilized eggs (Abdella et al., 1985). A higher proportion of male offspring in arrhenotocous parasitoid species may be caused by numerous factors, among them, e.g., mating success, age of mates, maternal crowding, mate competition. Furthermore, the host quality and density, superparasitism, microbial infection and abiotic factors may contribute to an increase in the number of males in a parasitoid population (e.g., Godfray, 1994; Fuester et al., 2003; West et al., 2005; Boulton et al., 2015; Nurkomar et al., 2021). Since parasitoids in the test cages with 2CM treatment and in control cages were exposed to the same mating conditions prior to the experiment, same densities (three parasitoid females of same age per cage), same host qualities and densities, and same abiotic factors, none of the above-mentioned factors is likely to have contributed to the observed changes in the composition of parasitoid progeny. In addition, the proportion of males in parasitoid progeny in the control cages was similar to our permanent rearing of *H. sylvanidis* (1 male to 3 females).

We suggest that the 2CM-induced search activity of the females negatively affected successful egg fertilization. The increased search activity might have reduced egg fertilization (i) by affecting the sperm release from the female's spermatheca storing the male's ejaculate after mating and/or (ii) by changing the quality of the sperm in the female's spermatheca. It is well known for insects that they can control the release of sperm from their spermatheca (e.g., Klowden, 2009). The 2CM-induced search activity might be linked with reduced release of sperm from the spermatheca. Interestingly, the biogenic amines octopamine and tyramine are known to affect both an insect's locomotory activity as well as sperm release (Avila et al., 2012; Selcho et al., 2012). Whether indeed 2CM can affect signaling by these biogenic amines remains to be analyzed by future studies. Alternatively or additionally, the 2CM-enhanced search activity might result in reduced sperm quality in the spermatheca because probably energy-taking, enhanced search activity might lead to changes in factors important for sperm maintenance in the spermatheca (Wolfner, 2011; Pascini and Martins, 2017). Another trade-off of intensive searching activity induced by synthetic kairomones might negatively affect the energy budget of parasitoid females by, e.g., reducing their fecundity and longevity.

If a parasitoid population produces more male offspring in the presence of synthetic kairomones, this shift in the population composition may negatively affected success of biological control by parasitoids because parasitoid females are needed to parasitize the host and reduce the host (pest) population (Heimpel and Lundgren, 2000). Thus, the attempt to improve biological control by application of 2CM needs to cope with the trade-off between 2CM-induced parasitization rate and an increasing parasitoid density as well as a 2CM-induced change in the sex distribution of

the parasitoid population. It needs to be considered that males are necessary to maintain a parasitoid population stable as the production of female parasitoid offspring requires fertilization of eggs in arrhenotecous species (Mayhew and Heitmans, 2000; Amante et al., 2017a). In some parasitoid species, male parasitoids can influence the offspring sex ratio to some extent, for example, by producing more female offspring to transfer the father's genetic contribution to the next generation (Henter, 2004; Shuker et al., 2006). Thus, the pendulum could swing back from a male-biased sex ratio in the other direction, thereby stabilizing the population. More research is needed to answer how application of 2CM would affect the population composition and stability of a *H. sylvanidis* population over time.

In summary, the release of attractive kairomones can improve the host search and thus host-finding success of *H. sylvanidis*. This approach might be very interesting for future biological control strategies of stored product pests, in particular in bakeries and mills where the use of pesticides is not tolerated. However, further studies are needed to test the potential of host-specific volatiles in larger experimental set-ups and over a longer period, especially to investigate possible effects on the sex allocation of the progeny. These results can contribute in various ways to advance the use of natural enemies in stored product protection and biologically based control of storage pest insects.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SA-S, MH, and BF contributed to the study conception and design. SA-S performed material preparation, data collection, analysis, and wrote the first draft of the manuscript which was revised by BF and MH. All authors commented on previous versions of the manuscript, read, and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1158081/full#supplementary-material

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Supporting data

The following supplemental data is available for this article:

Overview

Figure S1 (A) Design of the experimental set-up of the flight cage trial, (B) Detail sketch of a flight cage

Table S1 Detailed statistics on the comparison of behavioral responses of *H. sylvanidis* to infested wheat grist (IG) with different concentrations of (1) odors associated with *T. confusum* larvae and (2) odors associated with *Tribolium* adults

Table S2 Detailed statistics on how the additional application of 2CM influenced the (1) host search activity and efficacy, (2) parasitization and (3) offspring emergence of *H. sylvanidis* in a flight cage experiment (*N* = 40-47)

Release rate of 2CM from rubber septum: **Description of methods and results**

Figure S2 Gravimetrical analysis of the release rate of 2CM

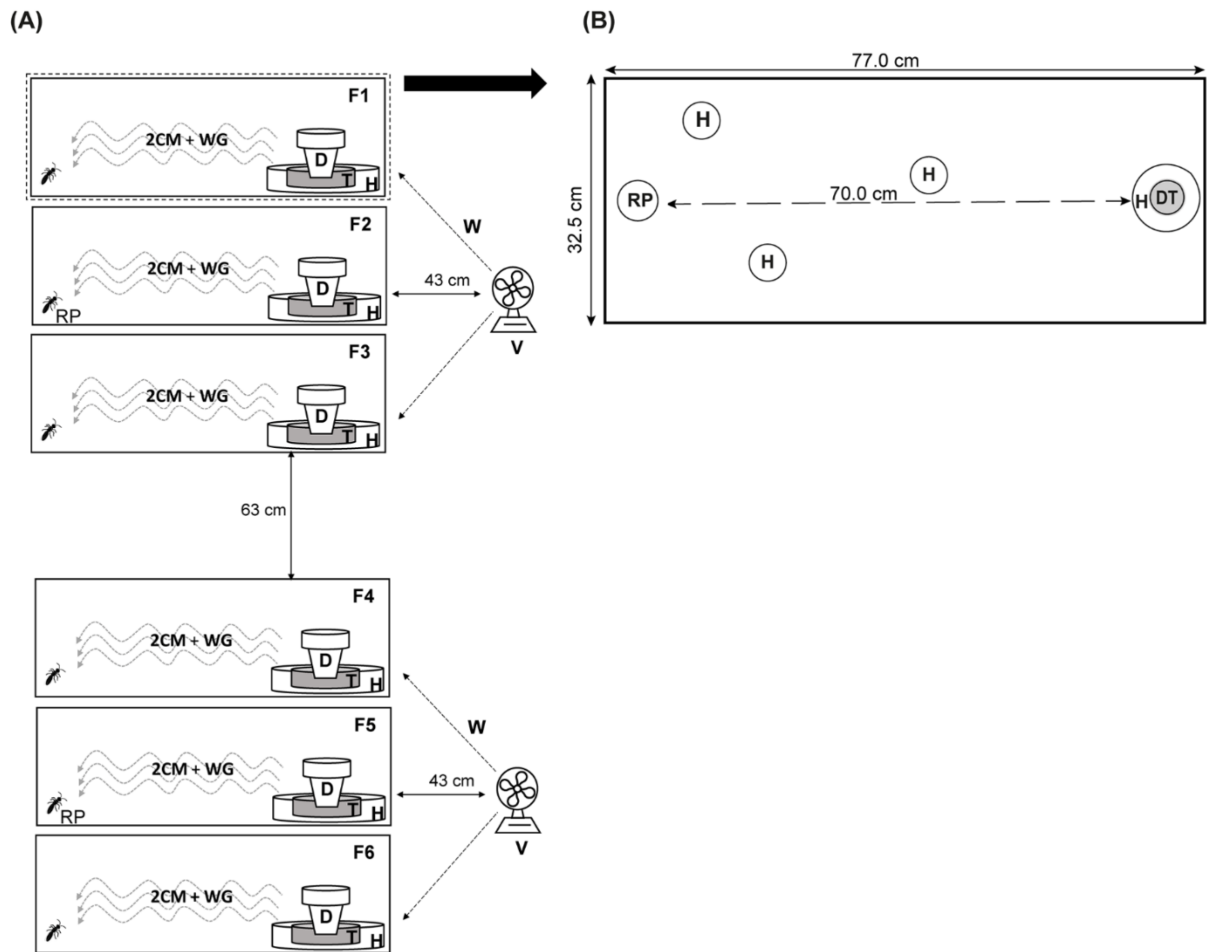

Figure S1 (A) Design of the experimental set-up of the flight cage trial, **(B)** Detail sketch of a flight cage. 2CM = two-component mix consisting of (*E*)-2-nonenal and 1-pentadecene, WG = odor of wheat grist, D = dispenser loaded with hexane (control) or 2CM (test), T= small Petri dish with 20 *Tribolium confusum* larvae feeding on wheat grist, F = flight cage, H = Petri dishes with five pipette tips as shelter sites for dislocated, paralyzed *T. confusum* larvae, V = ventilator, RP = release point of *Holepyris sylvanidis*, W = wind range.

Table S1 Detailed statistics on the comparison of behavioral responses of *H. sylvanidis* to non-infested wheat grist (NIG) or infested wheat grist (IG) with different concentrations of (1) odors associated with *T. confusum* larvae and (2) odors associated with *Tribolium* adults (N = 20-30)

Odors associated to	Statistical test	Comparison	χ^2-value	Df	*P*
(1) *T. confusum* larvae	One-sided binomial test	IG^2 vs. air	-	-	< 0.001
		$2CM_{low}{}^3$ vs. *n*-hexane	-	-	< 0.005
		$2CM_{low}{}^3 + NIG^1$ vs. NIG^1	-	-	< 0.001
		$2CM_{med}{}^4 + NIG^1$ vs. NIG^1	-	-	< 0.05
		$2CM_{high}{}^5 + NIG^1$ vs. NIG^1	-	-	< 0.05
	Test for equality of proportions	NIG^1 vs. IG^2	5.25	1	< 0.05
		$2CM_{low}{}^3$ vs. IG^2	0.45	1	> 0.05
		$2CM_{low}{}^3 + NIG^1$ vs. IG^2	< 0.001	1	1
		$2CM_{med}{}^4 + NIG^1$ vs. IG^2	9.08	1	< 0.05
		$2CM_{high}{}^5 + NIG^1$ vs. IG^2	15.63	1	< 0.001
(2) *Tribolium* adults	One-sided binomial test	$MBQ_{low}{}^6 + NIG^1$ vs. NIG^1	-	-	> 0.05
		$MBQ_{medium}{}^7 + NIG^1$ vs. NIG^1	-	-	> 0.05
		$MBQ_{high}{}^8 + NIG^1$ vs. NIG^1	-	-	> 0.05
		$MBQ_{very\ high}{}^9 + NIG^1$ vs. NIG^1	-	-	< 0.001
		$DMD^{10} + NIG^1$ vs. NIG^1	-	-	> 0.05
	Test for equality of proportions	$MBQ_{low}{}^6 + NIG^1$ vs. IG^2	7.05	1	< 0.01
		$MBQ_{med}{}^7 + NIG^1$ vs. IG^2	1.98	1	> 0.05
		$MBQ_{high}{}^8 + NIG^1$ vs. IG^2	7.05	1	< 0.01
		$MBQ_{very\ high}{}^9 + NIG^1$ vs. IG^2	21.62	1	< 0.001
		$DMD^{10} + NIG^1$ vs. IG^2	12.60	1	< 0.001

[1] NIG = non-infested wheat grist

[2] IG = infested wheat grist

2CM = Two-component mix ((*E*)-2-nonenal + 1-pentadecene, 1:1 in *n*-hexane) in three different concentrations (10 µL each applied):

[3] = 0.02 µg μL^{-1}, in total 0.2 µg

[4] = 0.2 µg μL^{-1}, in total 2 µg

[5] = 2 µg μL^{-1}, in total 20 µg

MBQ = Methyl-*p*-benzoquinone (in methanol) in four different concentrations (10 µL each applied):

[6] = 0.1 ng μL^{-1}, in total 1 ng

[7] = 1 ng μL^{-1}, in total 10 ng

[8] = 10 ng μL^{-1}, in total 100 ng

[9] = 100 ng μL^{-1}, in total 1000 ng

[10] DMD = Pheromone dispenser with 4,8-dimethyl decanal purchased by Trécé Incorporated (unknown concentration)

Table S2 Detailed statistics on how the additional application of 2CM influenced the (1) host search activity and efficacy, (2) parasitization and (3) offspring emergence of *H. sylvanidis* in a flight cage experiment (N = 40-47)

Evaluation parameter		Statistical test	
(1) *Host search activity and efficacy*	Number of dislocated *T. confusum* larvae after **1 h**	*Wilcoxon* rank-sum test	W = 1170, $P < 0.05$
	Number of dislocated *T. confusum* larvae after **24 h**	*Wilcoxon* rank-sum test	W = 1174, $P < 0.05$
	Number of dislocated *T. confusum* larvae after **48 h**	*Student t*-test	$T_{85} = 2.92$, $P < 0.005$
	Total number of dislocated *T. confusum* larvae (after **72 h**)	*Student t*-test	$T_{48} = 1.51$, $P > 0.05$
	Dislocation rate[1] (%)	*Wilcoxon* rank-sum test	W = 1169, $P > 0.05$
	Mean number of *T. confusum* larvae with host feeding marks	*Wilcoxon* rank-sum test	W = 1018, $P > 0.05$
	Efficacy of *H. sylvanidis*[2] (%)	*Wilcoxon* rank-sum test	W = 1173, $P < 0.05$
(2) Parasitization	Mean number of parasitized *T. confusum* larvae	*Wilcoxon* rank-sum test	W = 1165, $P < 0.05$
	Parasitization rate[3] (%)	*Wilcoxon* rank-sum test	W = 1207, $P < 0.05$
(3) Offspring emergence	Mean number of emerged *H. sylvanidis* females	*Wilcoxon* rank-sum test	W = 1209, $P > 0.05$
	Mean number of emerged *H. sylvanidis* males	*Wilcoxon* rank-sum test	W = 1248, $P < 0.05$
	Mean number of all emerged *H. sylvanidis* offspring individuals[4]	*Wilcoxon* rank-sum test	W = 1318, $P < 0.05$
	Mean overall emergence rate[5] (%)	*Wilcoxon* rank-sum test	W = 1267, $P > 0.05$
	Sex ratio	*χ^2 goodness of fit test*	$\chi^2(1) = 2.31$, $P > 0.05$

[1] Calculated on the number of dislocated *T. confusum* larvae

[2] Calculated on the number of dislocated *T. confusum* larvae and those larvae with host feeding marks

[3] Calculated on the number of offered *T. confusum* larvae (N = 20 per cage)

[4] Male and female offspring as well as those emerged *H. sylvanidis* offspring whose sex could not be identified

[5] Calculated on the total number of emerged *H. sylvanidis* offspring divided by the number of parasitized *T. confusum* larvae

Release rate of 2CM from a rubber septum

Methods

The release rates of both components of 2CM from a rubber septum were individually determined based on a gravimetric method described by Nielsen et al. (2019). This method relies on weight loss of evaporating, volatile components on the septum over time.

Natural rubber septa (9-10 mm outer diam., glass tubing, Sigma-Aldrich, Taufkirchen, Germany) were used as passive dispensers. Each dispenser was stuck to a lower part of a plastic Petri dish (50 mm diam.) using a heat glue gun. Prior to application of the kairomonal components, the rubber septa were placed for 24 h under a fuming hood to let any odors inherent to the glue, the dish or the dispenser itself evaporate.

Thereafter, each dispenser was weighted and then loaded with 1-pentadecene or (*E*)-2-nonenal. We applied a volume of 50 µL of either 1-pentadecene or (*E*)-2-nonenal onto a septum. These volumes correspond to 38.75 mg 1-pentadecene and 42.53 mg (*E*)-2-nonenal, respectively. The weight of each compound was calculated by its specific mass in 50 µL.

After solvent evaporation (5 min), the experiment was started by determining the weight of the loaded dispensers. Thereafter, weighing of dispensers was conducted at nine time points: at the day of application as well as one, three, six, seven, eight, nine, ten and thirteen days after application. The experiment took place at 22.5 ± 0.5°C and ambient humidity. In total, ten dispensers were used for each compound (1-pentadecene and (*E*)-2-nonenal).

To analyze the release rates of larval kairomones from rubber septa, we calculated the mean weight loss of each compound at each of the nine time points after application. To statistically compare whether the release rates of 1-pentadecene and (*E*)-2-nonenal differ significantly, we applied a linear mixed-effects model with a 6th degree polynomial. We used the “lmer()”-function of the “lme4”- package (Bates et al., 2015).

Results

Release kinetics of 2CM from a rubber septum

The release rate of 50 µL 1-pentadecene from a rubber septum differed significantly from the one of 50 µL (*E*)-2-nonenal (Figure S2, $\chi^2 = 1281.87$, df = 2, $P < 0.001$). For (*E*)-2-nonenal, the greatest weight loss was measured within two days after application on rubber septum. More than 20 µg were lost within a day. From the third day on, the compound was still released, but in low quantities. The decrease of (*E*)-2-nonenal within the observed period of thirteen days is described by the following equation: $Y = 4.63e^1 - 4.09e^1x + 2.06e^1x^2 - 4.93e^0x^3 + 6.07e^{-1}x^4 - 3.70e^{-2}x^5 + 8.82e^{-4}x^6$. In contrast, 1-pentadecene was continuously released in low amounts over the period of thirteen days but the release was not linear

(Figure S2). The decrease of this compound is described by the following equation: $Y = 3.95e^{1} - 2.71e^{0}x - 1.02e^{-1}x^{2} + 7.75e^{-2}x^{3} - 8.20e^{-3}x^{4} + 2.01e^{-4}x^{5} + 4.34e^{-6}x^{6}$.

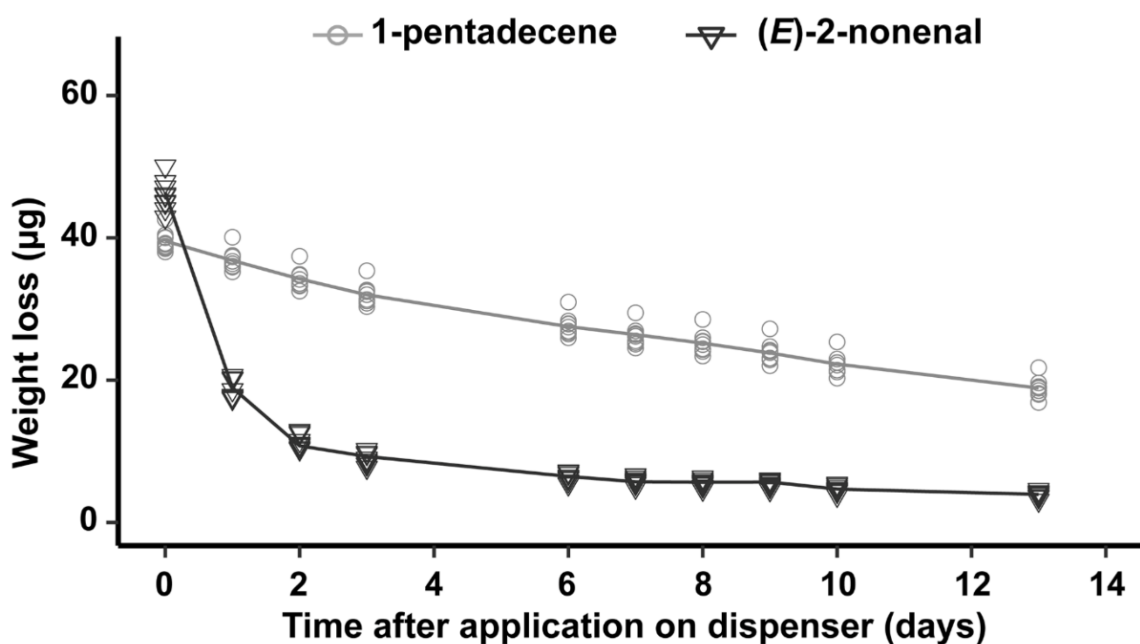

Figure S2 Gravimetrical analysis of the release rate of 50 µL 1-pentadecene (≙ 38.75 mg) and (*E*)-2-nonenal (≙ 42.53 mg) measured for 13 days (*N* = 10 per compound)

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Chapter 5:

General discussion

General discussion

My thesis focused on different factors determining the semiochemicals mediating host-searching behavior of the larval ectoparasitoid *Holepyris sylvanidis.* The results of my study examining how cuticular hydrocarbon (CHC) trails from *Tribolium confusum* larvae lose their kairomonal activity for *H. sylvanidis* over time are presented in Chapter 2. The study addressing the question whether the parasitoid uses a common pattern of CHCs to discriminate host species from non-host species is presented in Chapter 3. A further question was whether the additional application of host-specific kairomones can improve the host-searching success and parasitization rate of *H. sylvanidis* and thus, its efficiency in controlling *T. confusum*. The study of this question is presented in Chapter 4. A graphical abstract of these results is shown in Figure 1.

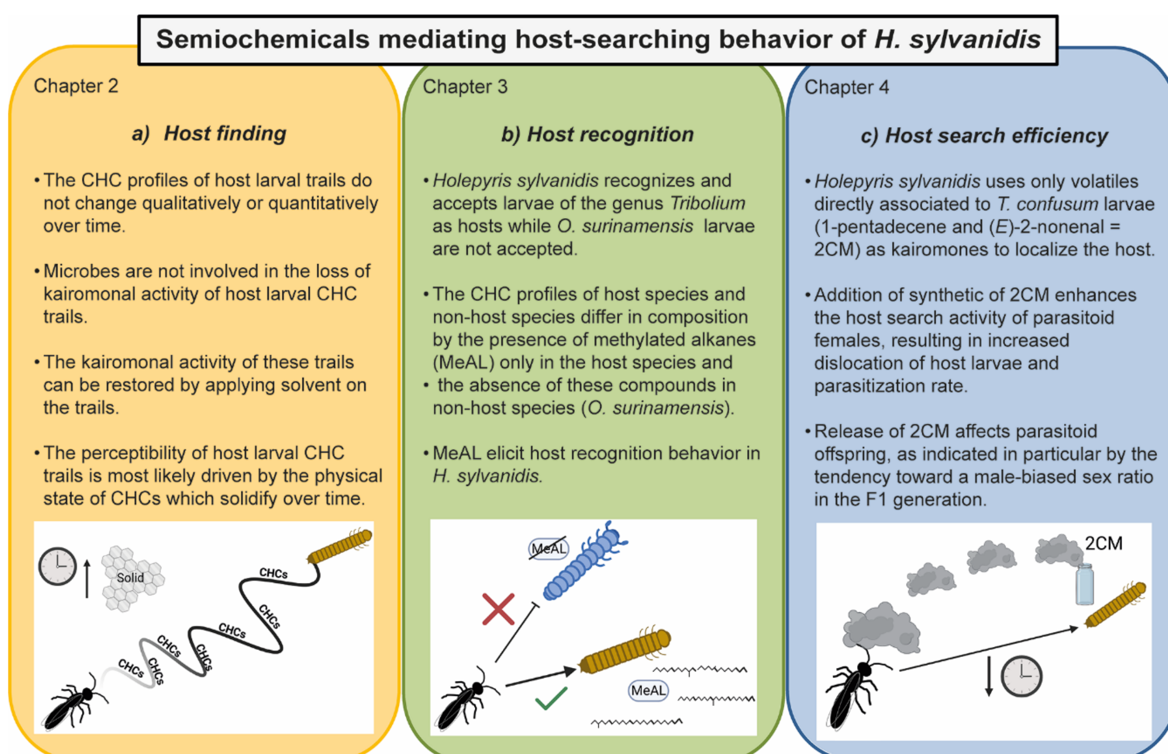

Figure 1 Graphical abstract of the main findings of this thesis on the semiochemicals mediating host-searching behavior of *Holepyris sylvanidis* regarding **a)** host finding (Chapter 2), **b)** host recognition (Chapter 3), and **c)** host search efficiency (Chapter 4). Abbreviations: CHC(s) = cuticular hydrocarbon(s), Solid = solidification processes of CHCs, MeAL = methyl-branched alkanes, 2CM = two component mix containing 1-pentadecene and (*E*)-2-nonenal, ratio 1:1). The image was created with BioRender.com

Prior to our study, it was assumed that the temporarily kairomonal activity of host CHC trails might be due to shifts in the composition of CHCs. But for larval *T. confusum* CHC trails, we demonstrated that the loss of informative activity of host trails was not linked to any microbial or other mediated chemical degradation of CHCs. The composition of CHC trails did not change qualitatively or quantitatively at different time points (0, 24, and 48 h after trail application) regardless of whether host trails were analyzed under sterile or non-

sterile conditions. In contrast, CHC trails elicited significantly less trail-following behavior in *H. sylvanidis* as time progressed. Our results of cryo-scanning electron microscopy (Cryo-SEM) showed that within two days, CHC trails gradually formed solid structures due to self-assembling processes, but these structures were reversible by hexane application. Parasitoid females followed re-activated 48-hour-old CHC trails just as when they encountered freshly laid ones. We suggested that the perceptibility of CHC trails over time was most likely driven by the physical state of CHCs (Figure 1a).

Previously, it was known that methyl-branched alkanes (MeAL) mediate intraspecific chemical communication in parasitoids. But we have revealed that MeAL are also involved in the chemical communication between different insect species. In contact bioassays, *H. sylvanidis* females successfully recognized larvae of different *Tribolium* species (*T. castaneum*, *T. confusum* and *T. destructor*) as hosts, whereas larvae of *Oryzaephilus surinamensis* were rejected. Larvae of the latter species were successfully recognized as hosts only when these larvae were extracted with a solvent and then treated with a sample of *T. confusum* larval CHCs. The main differences between the CHC profiles of host and non-host species were the presence of MeAL on the host cuticle (here *Tribolium* spp.) and the absence in the non-host (here *O. surinamensis*). Therefore, MeAL are contact kairomones which elicit host recognition behavior in *H. sylvanidis* (Figure 1b).

Previous studies have demonstrated that the application of synthetic, host-associated semiochemicals in the field can enhance the host-searching efficiency of parasitoids in controlling local pest populations. Our study revealed that the use of long-range attractants is also a promising method to improve biological control in warehouses and food processing facilities. *Holepyris sylvanidis* females were highly attracted to a mix of two key larval kairomones (1-pentadecene and (*E*)-2-nonenal; referred as 2CM) which were directly associated with the host larval stage of *T. confusum*. Interestingly, the behavioral response to 2CM was concentration dependent. In contrast, volatiles associated with *T. confusum* adults (methyl-*p*-benzoquinone and 4,8-dimethyldecanal) were not attractive for parasitoid females. In a flight cage experiment, parasitoid females removed significantly more beetle larvae from the initial *T. confusum* population and parasitized a significantly higher number of host larvae in the presence of 2CM compared to the control. Hence, significantly more parasitoid offspring emerged from host larvae in the test cages. This increased population size in F1 was due to a significantly higher number of male individuals. Our results show that the additional application of 2CM can improve the host-searching and parasitization success of *H. sylvanidis* but affects the sex distribution in parasitoid progeny (Figure 1c).

In the following sections, the experimental results of these studies will be linked to current knowledge on chemically mediated host search of parasitoids. The focus of the comparison will be primarily on semiochemicals that are known to influence the foraging behavior of generalist parasitoids over short- and long-range host search.

5.1. Physical traits of host-indicating CHCs determine their temporal perceptibility to parasitoids

Successful foraging behavior of parasitic wasps depends on the recognition of host-associated cues indicating the presence of a potential host. Therefore, these cues should be reliable in time and space to provide precise information about the current position of the host in a specific area for parasitoids. This is especially important for parasitoids specialized on a mobile host stage such as larvae which move freely in the habitat and thus, can constantly change the location (Vet and Dicke 1992; Vinson 1998; Steidle and Van Loon 2003; Hilker and McNeil 2008).

Parasitoids respond to various semiochemicals that continuously tune their foraging behavior (Vinson 1998). Over long distances, highly volatile, host-indicating cues that are released from plants in response to larval feeding (herbivore-induced plant volatiles, HIPVs) and to egg laying (oviposition-induced volatiles, OIPVs) of the host species can guide parasitoids to a potential host habitat. Furthermore, volatile cues emitted from host products (e.g., larval feces) can serve orientation of host-searching parasitoids (Steidle and Van Loon 2003; Hilker and Meiners 2006; van Oudenhove et al. 2017). Over short distances, low volatile chemicals often serve as host-indicating cues (Vinson 1998; Steidle and Van Loon 2003; Colazza et al. 2014). For instance, several parasitoids are known to recognize and follow trails that consist of long-chain hydrocarbons released from the cuticle of the host or other developmental stages of the host species (i.e., cuticular hydrocarbons, CHCs) (Howard and Flinn 1990; Howard et al. 1998; Colazza et al. 2009; Lo Giudice et al. 2011). A common feature of all these host-indicating cues is that they provide only temporary information about where to find a host, and therefore lose reliability for parasitoids over time (Rostás and Wölfling 2009; Holopainen and Blande 2013; Fürstenau and Hilker 2017).

From the ecological perspective, the time-limited availability of host-indicating cues helps parasitoids in decision making during host search. For example, parasitoids might not hunt a host that has already moved away, and thus are saving energy and time. Nevertheless, the mechanisms how host-indicating cues lose their informative activity are only partially understood. Douma et al. (2019) have shown that the physical properties of HIPVs determine their lifetime in the atmosphere and consequently, influence the

perceptibility by parasitoids. Upon release by plants, HIPVs (e.g., green leaf volatiles (GLVs) and terpenoids) immediately react with oxidizing agents present in the atmosphere such as ozone (O_3), nitrate radicals ($\cdot NO_3$) and hydroxyl radicals ($\cdot OH$). Especially, compounds with a high number of C-C double bonds (e.g., *β*-caryophyllene and linalool) have a short lifetime due to a fast degradation caused by reactions with atmospheric radicals (Atkinson and Arey 2003; Pinto et al. 2010; Holopainen and Blande 2013). In addition, GLVs and terpenoids can be completely degraded in the presence of high levels of O_3, which reduce their perceptibility for foraging parasitoids (Pinto et al. 2007 a, b). Due to different physical properties, HIPVs vary in the lifetime in the atmosphere, ranging from a few minutes to <24 h (Holopainen and Blande 2013). Most likely, the influence of HIPVs on the foraging behavior of parasitoids lasts as long as they can perceive these host-indicating cues.

In comparison, host larval trails containing long-chain CHCs elicit trail-following behavior in parasitoids over a longer period. Nevertheless, it has been shown that the kairomonal activity of CHC trails lasts for a maximum of three days (Rostás and Wölfling 2009; Fürstenau and Hilker 2017). The low volatility of CHC trails contrasts with the low persistence of these compounds which would be expected to be in the range of several days. It has been assumed that the loss of behavioral activity of CHCs trails might be due to changes in the CHC composition over time. One possible explanation might be degradation processes caused by oxidizing agents in the air or by microbes present on the substrate or released by host insects when depositing a trail (Napolitano and Juárez 1997; Collatz and Steidle 2008; Rostás and Wölfling 2009; Austin and Callaghan 2013; Pedrini et al. 2013). Another explanation could be that microorganisms, which live in symbiosis with herbivorous hosts, are able to actively interfere with the herbivore’s biosynthesis of CHCs by modulating the availability of CHC precursors and hence, shaping the host chemical profile (Engl and Kaltenpoth 2018; Sprenger and Menzel 2020). Qualitative and quantitative shifts in the CHC profile of herbivores can have a significant impact on interactions with conspecifics and mutualistic partners when CHCs that serve as recognition cues are affected (Engl and Kaltenpoth 2018; Hertaeg et al. 2021). In addition, the epicuticular wax layer of the plant surface might also modulate parasitoids’ perception of larval trails over time as shown in previous studies (Müller and Riederer 2005; Rostás et al. 2008; Colazza et al. 2009; Rostás and Wölfling 2009).

However, for *T. confusum* larvae, we showed that the temporary behavioral response of foraging parasitoids to CHC trails depends on the physical properties of the compounds involved (Chapter 2). The mechanisms how the behavioral activity of HIPVS and CHCs is lost in the course of time are most likely different. While reactions to atmospheric radicals lead to the complete degradation of HIPVs over time (Atkinson and

Arey 2003; Pinto et al. 2010), the CHC profile of larval *T. confusum* trails could be almost completely recovered 48 h after trail deposition and thus, was not subjected to oxidative decomposition. The fact that we did not detect qualitative or quantitative changes in the CHC composition of larval trails, underscores that factors other than degradation processes are involved in the temporarily informative value of CHC trails (Chapter 2).

Host trails of *T. confusum* larvae consist of a diverse blend of long-chain CHCs (linear alkanes, mono- and dimethyl-branched ones), differing in the melting temperatures (T_m) due to different carbon chain length and methylation (Gibbs and Pomonis 1995; Gibbs and Rajpurohit 2010, Chapter 2). Long-chain *n*-alkanes, but also some monomethyl-branched alkanes are known to have a high T_m and are solid at ambient temperatures. In contrast, dimethyl-branched alkanes are most likely liquid in this temperature range due to a low T_m (Gibbs and Pomonis 1995; Gibbs 2002; Brooks et al. 2015). Therefore, it is very likely that after trail deposition, CHC trails will begin to rearrange themselves and thus, gradually change from the liquid state into a solid state of compounds depending on the physical properties of CHCs present. Because of the self-assembling processes of CHCs, host trails form a solid-liquid matrix at ambient temperature range, likely become less accessible for host-searching parasitoids as the quantity of liquid CHCs continuously decreases. This assumption is supported by our results of cryo-SEM which showed the gradual solidification of CHC trails over a period of two days (Chapter 2).

Our results indicate that the temperature range, at which host trails are deposited, might play an important role for the speed of transition from liquid to solid phase of CHCs (Gibbs and Pomonis 1995; Gibbs 2002) and thus, the temporary accessibility of these compounds for foraging parasitoids. Under natural conditions, however, temperatures might fluctuate and lead to very different behavioral responses of parasitoids depending on the actual temperature. Low temperatures might accelerate the solidification of CHCs and result in an earlier loss of the kairomonal activity of host trails than observed in our study (48 h). On the contrary, high temperature could lead to the opposite effect by delaying the solidification of CHCs and therefore, inducing trail-following behavior in parasitoids for a longer period.

Nevertheless, the current temperature might not only affect the temporary kairomonal activity of host trails, but also their CHC composition. Insects are known to adjust the chemical compositions of the cuticle to differences in temperature by e.g., increasingly producing CHCs with a higher T_m (e.g., long-chain alkanes and monomethyl-branched alkanes) and thereby protecting themselves from desiccation (Menzel et al. 2017; Sprenger et al. 2018; Buellesbach et al. 2018). A temperature-dependent shift in the CHC profile of insects might significantly affect the CHC composition of host trails e.g., by increasing the

abundance of CHCs with a high T_m. Therefore, a higher proportion of these CHCs might still lead to a gradual solidification of CHC trails over time despite higher ambient temperatures. However, this raises the question of the kairomonal activity of these altered host trails for foraging parasitoids. A study of Wittke et al. (2022) has revealed that acclimation to different temperatures might interfere with nestmate recognition of ants but did not compromise their recognition ability. In contrast, Kárpáti et al. (2023) have recently shown that the adaptation of the CHC profile of morphs of *Drosophila suzukii* (Diptera: Drosophilidae) to cold winter conditions affects their mating success. On this basis, the response of parasitoids to altered host trails probably depends on whether behaviorally active compounds are still present in the CHC composition after acclimation. In particular, long-chain methylated alkanes could play a crucial role because these CHCs are involved in waterproofing the insect cuticle as well as in chemical communication of a wide range of different insect (Dani et al. 2001; Sugeno et al. 2006; Guédot et al. 2009; De Narbonne et al. 2016; Sakata et al. 2017; Menzel et al. 2019; Baumgart et al. 2022; Wittke et al. 2022).

5.2. Monomethyl-branched CHCs as general cues for host recognition of an oligophagous parasitoid species

As parasitoids perceive a plethora of different odor cues during host search, it is essential to identify those which are reliable indicators for a potential host. The type of semiochemicals used by foraging parasitoids depends on their diet specialization and their host species. Specialist parasitoids are expected to use highly plant- and host-specific cues, whereas generalist parasitoids should respond to more general cues released by different host species on different host plants or feeding substrates. The use of general host-indicating semiochemicals may improve the host search by generalists under fluctuating environmental conditions e.g., when the occurrence of potential host species varies temporally and spatially (Vet and Dicke 1992; Steidle and Van Loon 2003; van Oudenhove et al. 2017). One example for such general cues are green leaf volatiles which are exploited by numerous generalist parasitoids for host habitat location and host location (e.g., Whitman and Eller 1990; Hoballah and Turlings 2005; Ngumbi et al. 2009; Ngumbi and Fadamiro 2012). In addition to plant-derived odors, highly volatile, host-specific kairomones (e.g., sex pheromones or volatiles emitted by host feces) can also serve as general cues for foraging parasitoids (Steidle and Ruther 2000; Steidle et al. 2001b, a; Schöller and Prozell 2002).

In contrast, little is known about whether host-specific kairomones with lower volatility are also used as general cues by foraging generalist parasitoids. These could be methylated alkanes (MeAL), which are abundant on the insect cuticle and influence the

differential behavior of different taxa (Howard and Blomquist 2005; Blomquist and Bagnères 2010). For social insects, MeAL are known to mediate the recognition of (nest) mates, kin, and mutualistic partners (Dani et al. 2001; Guerrieri et al. 2009; De Narbonne et al. 2016; Sakata et al. 2017). Even though MeAL have been already described as contact sex pheromones for species belonging to the parasitoid taxa of the Encrytidae and Pteromalidae (Steiner et al. 2005, 2007; Ruther et al. 2011; Ablard et al. 2012; Kühbandner et al. 2012b), it has been unknown prior to this thesis whether MeAL are involved in parasitoid-host interactions.

We were able to fill this knowledge gap by demonstrating that MeAL serve as host recognition cues and thus, contribute to the chemical communication between a bethylid parasitoid and several host species (Chapter 3). According to literature, the host range of *H. sylvanidis* includes different stored-product pest beetles belonging to the genera of Cucujidae, Tenebrionidae, and Silvanidae (Evans 1969; Hagstrum and Subramanyam 2009; Amante et al. 2017b). Our results showed clearly that the presence of MeAL on the host cuticle is crucial to elicit host recognition behavior in *H. sylvanidis*. Therefore, different *Tribolium* species were successfully recognized and accepted as hosts because they had a similar pattern of MeAL on their cuticle. In contrast, MeAL was absent on the cuticle of *O. surinamensis* larvae which in turn could not evoke host recognition behavior.

The composition of the CHC profiles of the host species were species-specific but nine MeAL were present on the cuticle of all *Tribolium* species (Chapter 3). Additionally, seven of these nine MeAL were also found in naturally laid trails of *T. confusum* larvae, inducing trail-following behavior in *H. sylvanidis* (Fürstenau and Hilker 2017). Therefore, we proposed that this group of seven monomethyl-branched alkanes (3-MeC25, 11-/13-MeC27, 3-MeC27, 12-/13-/14-MeC28, hereafter referred to as MoMeAL group) most likely act as contact kairomones for host recognition and perhaps also for trail-following behavior.

If *H. sylvanidis* uses ubiquitous compounds as general cues for host recognition, the question arises whether other beetle species could also be considered as potential hosts if compounds from the MoMeAL group are present on their cuticle. This question is of interest for the application of the parasitoid in warehouses and food processing facilities since *Tribolium* spp. frequently co-occur with other pest beetles which may also belong to the family of Tenebrionidae (Hagstrum and Subramanyam 2009). Interestingly, *H. sylvanidis* responded differently to larvae of two tenebrionid species in contact bioassays. Live larvae of the broad-horned beetle, *Gnathocerus cornutus*, were successfully recognized and accepted as hosts. In addition, the host recognition rate of *G. cornutus* larvae and the mean searching time until successful host recognition were not significantly different to those of

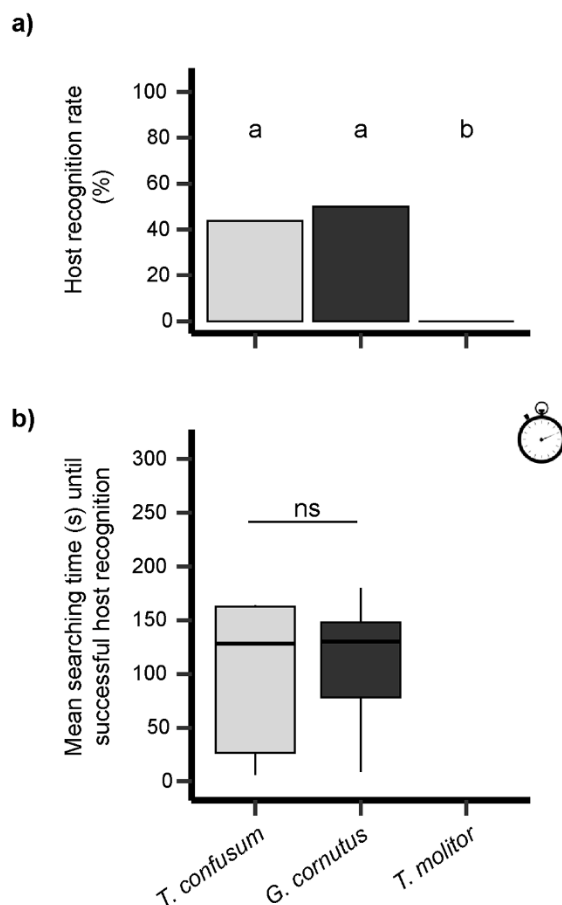

Figure 2 Behavioral responses of female *H. sylvanidis* to live larvae of different species of the Tenebrionidae family (*T. confusum*, *G. cornutus*, and *T. molitor*; *N* = 16 per species, max. observation time = 300 s). **a)** Host recognition rate (100% ≙ 16 successful host recognition events per beetle species) was analyzed by the test for equality of proportions followed by pairwise comparison of proportions with *Bonferroni-Holm* correction. Different letters above the bars indicate significant differences at $P < 0.05$. **b)** Mean searching time until successful host recognition was analyzed for *T. confusum* and *G. cornutus*, but not for *T. molitor* as larvae of the latter species were rejected as hosts by parasitoids. Data were statistically analyzed by *Whitney-Mann* U test (ns = not significant, $P > 0.05$)

T. confusum larvae. In contrast, larvae of the yellow mealworm, *Tenebrio molitor*, were rejected (Figure 2a + b).

A comparison of the CHC profiles of *G. cornutus* and *Tribolium* spp. showed that three compounds of the MoMeAL group (3-MeC25, 11-MeC27, and 3-MeC27) were present on the cuticle of all larvae, and 11-MeC27 was as the predominant one (Lane et al. 2016, Chapter 3). In contrast, only minute concentrations of 3-MeC25 and 3-MeC27 were detectable on the cuticle of *T. molitor*, whereas internally methyl-branched alkanes were absent (Lockey 1978; Ferreira-Caliman et al. 2012).

These results show clearly that other beetle species than *Tribolium* spp. can also induce host recognition behavior in *H. sylvanidis* when monomethyl-branched alkanes are present on the beetle cuticle. But these results also imply that a smaller number of compounds than the MoMeAL group are involved in the mediation of host recognition behavior. The presence of 3-MeC25, 11-MeC27 and 3-MeC27 in the CHC profile of

G. cornutus seems to be sufficient to evoke a behavioral response in *H. sylvanidis.* On the other hand, the rejection of *T. molitor* larvae in contact bioassays might be due to an incomplete MoMeAL group, probably stressing the importance of internally methyl-branched alkanes as host recognition cues.

Overall, the results presented in Chapter 3 show that the investigation of the semiochemicals mediating host-finding behavior of *H. sylvanidis* not only broadened our knowledge of the role of MoMeAL mediating parasitoid-host interactions but also led to new insights into the host range of the parasitoid.

Prior to this work, it was not known that *T. destructor* and *G. cornutus* are possible host species of *H. sylvanidis.* Furthermore, *O. surinamensis* was previously considered a host species but this could not be confirmed by our studies. Consequently, at least for our laboratory breeding line of the parasitoid at the JKI, I suggest that *H. sylvanidis* is an oligophagous parasitoid species that uses a specific set of 3-methyl and internally methyl-branched alkanes as general cues for host recognition.

5.3. Potential effects caused by shifts in host diet on the behavioral activity of monomethyl-branched CHCs

Under natural conditions, abiotic and biotic environmental factors (e.g., diet and temperature) might significantly affect the chemical communication of insects (Otte et al. 2018; Chapter 2). For example, the colonization of a new host plant or feeding substrate can lead to differences in the nutrient supply for the biosynthesis of CHCs (Blomquist 2010; Otte et al. 2015). Thus, a change in diet can induce qualitative and quantitative differences in CHC composition, within a short period of one generation or within as little as two weeks (Buczkowski et al. 2005; Kühbandner et al. 2012a; Geiselhardt et al. 2012; Khidr et al. 2013). If shifts in the CHC profiles of insects lead to different CHC phenotypes, the interactions between insects might be significantly altered. It has been already shown for several ant species that due to diet-dependent CHC phenotypes, individuals were able to distinguish between conspecifics reared on the same diet and those on an alternative diet. As a result, ants showed a higher aggressiveness to conspecifics with a different CHC phenotype (Liang and Silverman 2000; Buczkowski et al. 2005; Sorvari et al. 2008; Vonshak et al. 2009). Furthermore, the feeding on different host plants might influence the mate choice of species when e.g., males prefer to mate with females which feed on the same host plant and have the same CHC phenotype (Geiselhardt et al. 2009, 2012; Xue et al. 2016). If the differences in the CHC composition promote assortative mating, it may initiate ecological speciation (Otte et al. 2018).

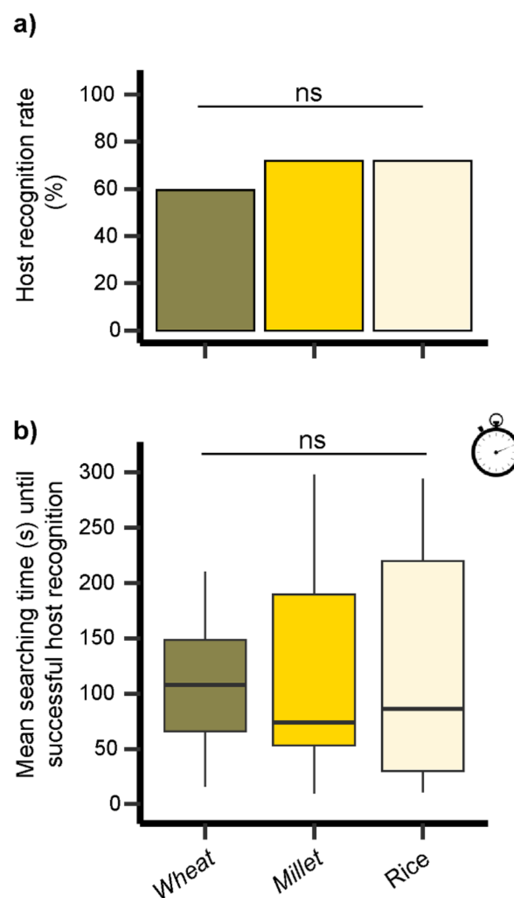

Figure 3 Behavioral responses of female *H. sylvanidis* to *T. confusum* larvae which were reared on wheat, millet, or rice (N = 32 per feeding substrate, max. observation time = 300 s). **a)** Host recognition rate (100% ≙ 32 successful host recognition events per feeding substrate) was analyzed by the test for equality of proportions followed by pairwise comparison of proportions with *Bonferroni-Holm* correction. **b)** Mean searching time until successful host recognition was analyzed by *Kruskal-Wallis* test (ns = not significant at $P < 0.05$)

In contrast, it is still poorly understood to which extent diet-mediated changes in the chemical composition of the host cuticle can have an impact on the behavior of individuals of higher trophic levels (e.g., the host search of parasitoids). Larvae and adults of *Tribolium* spp. and *G. cornutus* are generalist pest beetles, infesting a broad range of different stored plant products in the food processing industry worldwide (Hagstrum and Subramanyam 2009). Therefore, the composition of the CHC profiles of these pest beetles is expected to vary phenotypically depending on the type of stored and processed commodity they had fed on. This raises the question of whether the MoMeAL group remains a reliable host-indicating cue for *H. sylvanidis* despite possible diet-related effects on the CHC profile of host species.

To address this question, individuals of *T.* confusum were kept on three different feeding substrates (wheat, millet, and rice grist) for at least five generations. Then, chemical analysis of crude larval extracts and contact bioassays were performed similar to the protocol described in Chapter 3. In the following, the terms “wheat line”, “millet line”, or “rice

line” will be used when referring to beetle larvae that were reared on wheat, millet, or rice grist, respectively.

GC-MS analysis revealed that the MoMeAL group was still present in the CHC profile of *T. confusum* larvae kept on rice, millet, or wheat. But the ratios of the compounds within the MoMeAL group varied between the different feeding lines, resulting in three diet-dependent phenotypes. These findings are in accordance with Geiselhardt et al. (2012), who found a similar diet-mediated effect on the CHC composition of the mustard beetle, *Phaedon cochleariae* (Coleoptera: Chrysomelidae). The different, diet-related CHC phenotypes significantly affected the mate recognition system of *P. cochleariae*, leading to assortative mating (Geiselhardt et al. 2012). In contrast, the different phenotypes of the MoMeAL group did not alter the interaction between *H. sylvanidis* and *T. confusum* larvae of the different feeding lines. In contact bioassays, parasitoid females successfully recognized *T. confusum* larvae regardless of whether the beetle larvae were reared on wheat, millet, or rice (Figure 3a). Furthermore, the host recognition rate and the mean searching time until successful host recognition did not significantly differ among the three feeding lines (Figure 3b). These results clearly show that the different diet-phenotypes did not compromise the kairomonal activity of the MoMeAL group to elicit host recognition behavior in the parasitoid.

Nevertheless, *H. sylvanidis* females tended to recognize a higher number of *T. confusum* larvae of the millet and rice line as hosts compared to beetle larvae of the wheat line (Figure 3a). The differences in the behavioral response of parasitoids are most likely due to the occurrence of different phenotypes of the MoMeAL group, probably caused by different nutrient supply provided by the feeding substrates. For the biosynthesis of 3-methyl- and internally branched alkanes, the availability of the amino acids valine, isoleucine, and methionine as well as vitamin B_{12} is important. These nutrients are precursors for methyl-malonyl-CoA that gives rise to 3-methyl- and internally branched alkanes (Blomquist and Bagnères 2010; Blomquist and Ginzel 2021). In particular, millet is known to have a high content of essential amino acids and B-complex vitamins (Saini et al. 2021; Bangar et al. 2022; Mahajan et al. 2023). It is very likely that feeding on millet and rice grist might enhance the production of CHC types, leading to a higher abundance of the MoMeAL in the CHC profile of *T. confusum* larvae compared to those of the wheat line. A higher abundance of these compounds on the host cuticle possibly reinforced the perceptibility of MoMeAL group for *H. sylvanidis.*

It is still unclear why the MoMeAL group is preserved on the host cuticle (here *T. confusum*) under different environmental conditions (here different feeding substrates) when the biosynthesis of these compounds is likely to come at great metabolic cost

(Blomquist and Ginzel 2021) and the presence of these compounds might only benefit an antagonist, i.e., *H. sylvanidis.* One possible explanation could be that compounds of the MoMeAL group might be involved in the intraspecific communication of *Tribolium* species. Some compounds in the MoMeAL group are known to act as contact sex pheromones in different insect taxa (Sugeno et al. 2006; Guédot et al. 2009; Silk et al. 2011; Kühbandner et al. 2012b, 2013). In addition, Lo et al. (2023) have recently revealed that *T. castaneum* recognizes the immune status of conspecifics by MeAL. Therefore, the presence of compounds belonging to the MoMeAL group on the cuticle of *Tribolium* spp. could be genetically determined and thus, highly robust serving the maintenance of intraspecific chemical communication.

Previous studies have already shown for the generalist parasitoid *Lariophagus distinguendus* (Hymenoptera: Pteromalidae) that for host recognition, this parasitoid species uses general cues persisting under different environmental factors (different host species and host plant seeds). A specific blend of sterols, tocopherols and tocotrienols was detected in the odor bouquet of larval feces of taxonomically different host species feeding on different seeds of taxonomically different host plants (e.g., *Sitophilus granarius* (Coleoptera: Curculionidae) – wheat and rice, *Rhyzopertha dominica* (Coleoptera: Bostrichidae) on wheat) (Steidle and Ruther 2000; Steidle et al. 2001a, b). Likewise, the egg parasitoid, *Trichogramma evanescens* (Hymenoptera: Trichogrammatidae) uses the main component of the pyralid sex pheromone (*Z, E*)-9,12-tetra-decadenyl acetate (TDA) for host location (Schöller and Prozell 2002). TDA has been already detected in different pyralid species infesting different stored products (e.g., *Plodia interpunctella* on a mix of wheat and dried fruits, *Ephestia elutella* on cacao) (Kuwahara et al. 1971; Kuwahara and Casida 1973; Zhu et al. 1999; Ryne et al. 2006; Trematerra et al. 2011). Interestingly, *L. distinguendus*, *T. evanescens*, and *H. sylvanidis* are natural enemies of stored-product pests that attack a broad range of post-harvest plant products. Using general cues for host search may facilitate the adaption of polyphagous and oligophagous parasitoids to new environmental conditions. This may represent an advantage for the release of these three parasitoid species in biological control approaches against different pest species in diverse storage facilities.

5.4. Use of host-specific semiochemicals in the biological control of stored-product pests

In recent decades, the release of natural enemies (e.g., parasitoids) as an alternative control strategy to conventional chemical pesticides has been increasingly promoted by the public and policymakers, resulting in the use of augmentative biological control (ABC) on

estimated 30 million hectares in 2015 alone (van Lenteren 2012; European Commission 2015, 2022; van Lenteren et al. 2018). The foraging behavior of parasitoids, however, is known to be affected by various environmental factors, which may be crucial for the success of biological control programs in agroecosystems. The additional application of synthetic, long-range attractants is a promising approach to manipulate the host-searching behavior of parasitoids in order to improve their effectiveness in suppressing pest populations under field conditions (Meiners and Peri 2013; Ayelo et al. 2021). Field application of synthetic semiochemicals (e.g., HIPVs and sex pheromones of pest species) in the presence of released or naturally occurring parasitoids has been already reported to enhance the host finding of various parasitoid species belonging to the taxa of Aphidiinae, Braconidae, Encyrtidae, Mymaridae, and Trichogrammatidae (Lewis et al. 1982; Glinwood et al. 1998; Franco et al. 2008, 2011; Uefune et al. 2012; Ingrao et al. 2019; Xiu et al. 2019). For instance, *Anaphes iole* (Hymenoptera: Mymaridae) parasitized more eggs of the tarnished plant bug, *Lygus lineolaris* (Heteroptera: Miridae) in the presence of two synthetic HIPVs, *α*-farnesene and (*Z*)-3-hexenyl acetate (Williams et al. 2008). In addition, exogenous application of a plant hormone, jasmonic acid (JA), is known to indirectly affect the behavior of parasitoids by increasing the biosynthesis and release of plant volatiles (Thaler 1999; Lou et al. 2005). This can lead to a higher abundance of parasitoids and thus, a twofold higher parasitization of pest species as shown in a field study by Lou et al. (2005). These findings emphasize the new perspectives for the biologically based pest control opened by using long-range attractants.

In contrast to open agroecosystems (e.g., crop fields and orchards), warehouses and food processing facilities are more closed systems and might represent more favorable locations for the release of parasitoids because of more stable environmental conditions and structural elements preventing parasitoids from leaving. Consequently, the use of parasitoids could have the same potential for pest control in storage environments as they have in greenhouses (Schöller et al. 1997; Flinn and Schöller 2012).

Prior to this thesis, however, it was not yet known how and to which extent additionally applied semiochemicals might affect the host-searching behavior of parasitoids in storage environments. In Chapter 4, we demonstrated by a 72-h lasting flight cage experiment that *H. sylvanidis* females exhibited an increased host-searching activity in the presence of 2CM. Parasitoid females dislocated significantly more paralyzed *T. confusum* larvae from the initial population within 48 h and thus, parasitized significantly more host larvae after 72 h compared to the control cages. Therefore, our results are consistent with previous studies showing that the presence of host-associated volatiles can facilitate the host search of parasitoids by attracting and guiding them to the close vicinity of a potential

host. This can lead to an increased host-finding and parasitization success, thus improving the performance of parasitoids in controlling pest populations (e.g., Lewis et al. 1982; Williams et al. 2008; Uefune et al. 2012; Xiu et al. 2019).

Nevertheless, the 2CM-based manipulation of the host-searching behavior of *H. sylvanidis* has been only successfully demonstrated under laboratory conditions, whereas (field) studies investigating possible effects of 2CM on the searching activity of parasitoid females in storage sites and the food processing industry are still missing (Chapter 4). These studies under more natural conditions are essential to assess the potential of 2CM to enhance the efficiency of *H. sylvanidis* as biological control agent of pest insects infesting stored and processed plant products.

Several studies have already pointed out the difficulty of transferring findings on the chemical ecology of parasitoids obtained in the laboratory situations to field situations (Roland et al. 1995; see references in Kaplan 2012 or in Meiners and Peri 2013). For example, (*E*)-2-hexenal, a component of the metathoracic glandular secretion of stink bugs, has been described as highly attractive for the egg parasitoids *Telenomus podisi* and *Trissolcus basalis* (Hymenoptera: Platygastridae) in bioassays under laboratory conditions. But the application of (*E*)-2-hexenal in a soybean field increased only the abundance of *Trissolcus* spp. but did not affect the parasitism rate of host eggs of the brown stink bug, *Euschistus heros* (Hemiptera: Pentatomidae) (Laumann et al. 2009; Vieira et al. 2014). There are several explanations for the discrepancy in behavioral responses of parasitoids to host-associated volatiles in laboratory experiments and field trials.

One possible explanation is the choice of volatiles that has a great impact on which species will be attracted and to what extent (Kaplan 2012). Here, it should be considered that compounds, which attract the parasitoids, are often either derived from the intraspecific chemical communication of host species (e.g., sex pheromones) or plant-derived volatiles (e.g., HIPVs) (Vet and Dicke 1992; Steidle and Van Loon 2003). Therefore, the application of behaviorally modifying compounds on a large field scale may affect not only the parasitoid’s population dynamics (mass-reared or naturally occurring ones) but also the interactions of other insect species. This is because all interactions between insects are linked to a certain extent with other members in the arthropod community. When synthetic attractants are applied to alter the behavior of individuals of the third trophic level (parasitoids and predators), it may also have a significant impact on the behavior of individuals of the other trophic level (Kaplan 2012; Meiners and Peri 2013; Ayelo et al. 2021). For instance, one of the most field-tested HIPVs, methyl salicylate (MeSA), is known to be a broad-spectrum attractant, increasing the abundance of key beneficial insect groups including bugs, lady beetles, lacewings, syrphids, and parasitoids in the field (Rodriguez-

Saona et al. 2011). However, using synthetic MeSA in the field can also cause unintended effects, for example by attracting untargeted herbivorous pest species and hyperparasitoids (Orre et al. 2010; Orre-Gordon et al. 2013). Other ubiquitous HIPVs are likely to have similar effects on multitrophic interactions within the arthropod community. That is why the enhanced activity of parasitoids may be surpassed by increased herbivore abundance or occurrence of hyperparasitoids depending on the selected volatiles that guide parasitoid behavior.

In our flight cage experiment, we used a host-specific mix of two key larval kairomones (2CM) to successfully enhance the performance of *H. sylvanidis* as biological control agent of *T. confusum* (Chapter 4). Both compounds, (*E*)-2-nonenal and 1-pentadecene, are components of fecal odor released from *T. confusum* larvae (Fürstenau et al. 2016). But the latter is also known to be released as part of the aggregation pheromonal blend by female and male adults of *Tribolium* species (Keville and Kannowski 1975; Arnaud et al. 2002; Verheggen et al. 2007). Đukić et al. (2021) have recently shown that the response of *Tribolium* adults to 1-pentadecene is most likely concentration-dependent. Low concentrations of 1-pentadecene were highly attractive, whereas high concentrations were repellent (Đukić et al. 2021). Future studies need to elucidate how the additional application of 2CM for improving the efficiency of *H. sylvanidis* affects the abundance of the host *Tribolium*.

Another explanation for the differences in the parasitoid's response to synthetic semiochemicals in laboratory and field experiments is that the controlled environment in laboratory can only partially mimic field conditions. The chemical complexity in the habitat of pest species can impede the efficient use of these compounds by parasitoids. Host-indicating volatiles can be highly variable in time and space due to abiotic and biotic environmental factors, or they may become masked by the background odor of the habitat (Hilker and McNeil 2008; Wäschke et al. 2013; Meiners and Peri 2013; Chapter 2, Chapter 5.1). Interestingly, volatiles of the background odor (here the feeding substrate of the host species = non-infested wheat grist) can also increase the attractiveness of host-associated compounds (e.g., 2CM) as shown for *H. sylvanidis* in a previous study (Fürstenau et al. 2016). But the enhancing effect of habitat-related volatiles on the perception of 2CM by parasitoid females appears to be concentration-dependent. Regardless of the presence or absence of non-infested wheat grist, only the lowest concentration of 2CM ($2CM_{low}$) was attractive for *H. sylvanidis* females, while the highest concentration of 2CM was even repellent (Chapter 4). The most attractive concentration of 2CM (= $2CM_{low}$) is equivalent to the quantities of feces released by 100,000 4th instar *T. confusum* larvae and thus, indicates a very high host density (Fürstenau et al. 2016, Chapter 4). Therefore, higher concentrations

of 1-pentadecene and (*E*)-2-nonenal in 2CM than $2CM_{low}$ probably warn foraging parasitoid females of negative effects on their offspring and trigger avoidance behavior.

Several studies have already drawn the attention to the fact that applying synthetic host-associated volatiles at higher concentrations in the field than those naturally emitted by pest species or plants, might induce differential effects in foraging parasitoids (see references in Meiners and Peri 2013 or Vosteen et al. 2016). On the one hand, this method may facilitate the detection of additionally applied volatiles over long distance and raising the abundance of parasitoids and thus, the parasitization rate of pest species within a specific area (e.g., Glinwood et al. 1998; Williams et al. 2008; Xiu et al. 2019). On the other hand, using synthetic volatiles at high concentrations may disturb the host search of attracted parasitoids if these compounds interfere with kairomones naturally emitted by the host, preventing the parasitoids from detecting the exact location of the host. This may lead parasitoids to follow false trails which will waste their time and energy while searching in an area where there are no hosts. As a consequence, parasitoids might adjust their host-searching behavior by responding less to synthetic semiochemicals because these compounds have previously attracted them without any reward. In these cases, manipulating the behavior of parasitoids would be ineffective or counterproductive (Schöller 1998; Meiners and Peri 2013). This could also explain why in some field studies an enhancing effect of synthetic volatiles on the attraction of parasitoids, but not on the parasitization success, was found (e.g., Vieira et al. 2014; Zhao et al. 2022).

For the first time, we have found some indication that the use of synthetic kairomones may not only affect the host-searching behavior of *H. sylvanidis* but also the sex ratio of parasitoid offspring in the next generation (F1). Using 2CM resulted in a significantly increased number of emerged male offspring individuals with a constant number of female ones. This resulted in an altered population composition (Chapter 4). In arrhenotokous parasitoid species such as *H. sylvanidis*, male individuals are needed for the egg fertilization and thus, contribute to maintain a stable parasitoid population (Mayhew and Heitmans 2000; Amante et al. 2017a). Nevertheless, parasitoid females are required for the parasitization of the host and suppression of host (pest) populations (Heimpel and Lundgren 2000). It is not yet clear how a male-dominated sex ratio in the F1 generation of *H. sylvanidis* affects the population stability of the parasitoid on the long run. Furthermore, little is known whether other behaviorally modifying compounds might induce similar effects in the other parasitoid species. If so, any attempt to improve the performance of the parasitoids by applying synthetic, host-associated volatiles might need to cope with the tradeoff between an increased parasitization rate and increased parasitoid density as well as an altered sex distribution of parasitoid offspring. This might significantly affect the success of biological

control on the long term and needs to be considered particularly in conservative biological control where the use of synthetic volatiles directly intervenes in the dynamics and stability of natural parasitoid populations.

5.5. General conclusion and final remarks

This dissertation thesis provides several new insights into the role of semiochemicals that are relevant for the host search by the ectoparasitoid *H. sylvanidis*. It was shown that the loss of the kairomonal activity of host larval trail over time is due to the physical properties of CHCs present in the trails. The host recognition behavior of the parasitoid is mediated by a group of monomethyl-branched alkanes (MoMeAL) common on the cuticle of different host species; their presence is robust to environmental factors such as the diet of the pest species, however, their quantities may vary with the diet of the host. Furthermore, the additional application of synthetic kairomones can enhance the host-searching activity of parasitoid females, leading to a higher host-finding and parasitization success and thus, improving the efficiency of *H. sylvanidis* in controlling *T. confusum*. These results highlight that an in-depth knowledge of the chemically mediated host-searching behavior of *H. sylvanidis* can not only provide new application opportunities (e.g., by the detection of new host species) but also improved performance of parasitoids in biological pest control.

However, the results of this doctoral thesis raise new questions about the role of semiochemicals involved in parasitoid-host interactions. Our results suggest that the MoMeAL most likely serve as general cues on the short range and thus, enables *H. sylvanidis* to distinguish between host and non-host species under different environmental conditions. In contrast, it is not known whether the components of the 2CM, 1-pentadecene and (*E*)-2-nonenal, are general cues for the host location on the long range. Using general host-indicating cues for host location may help parasitoids (released in augmentative biological control) to adapt to different storage environments without compromising the performance of parasitoids as biological control agent. Furthermore, parasitoids are known to learn specific compounds when their perception is associated with a specific reward (e.g., a suitable host for oviposition) (Giunti et al. 2015). Hence, the combination of the additional application of synthetic host-associated volatiles and release of parasitoids which have been trained on these volatiles prior to the release, might be a further promising approach but has not yet been tested. The results can contribute in various ways to advance the use of natural enemies in stored-product protection and biologically based control of storage pest insects.

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