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https://orcid.org/0000-0002-2015-8679

VILNIUS UNIVERSITY
STATE SCIENTIFIC RESEARCH INSTITUTE NATURE RESEARCH CENTRE

Gabrielė Bumbulytė-Žukevičienė

Search for Environmentally Friendly Compounds for Behavioral Control of *Tenebrio molitor* L.

DOCTORAL DISSERTATION

Natural Sciences,
Ecology and Environmental Sciences N 012

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Academic supervisor – Prof. Habil. Dr. Vincas Būda (State Scientific Research Institute Nature Research Centre, Natural Sciences, Ecology and Environmental Sciences – N 012).

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Chairman – Prof. Dr. Algimantas Paulauskas (Vytautas Magnus University, Natural Sciences, Ecology and Environmental Science – N 012)

Members:
Prof. habil. Dr. Eugenija Kupčinskienė (Vytautas Magnus University, Natural Sciences, Botany – N 013),
Dr. Rasa Bernotienė (State Research Institute Nature Research Centre, Natural Sciences, Ecology and Environmental Science – N 012),
Dr. Irena Nedveckytė (Vilnius University, Natural Sciences, Ecology and Environmental Science – N 012),
Dr. Teodora Toshova (Institute of Biodiversity and Ecosystem Research Bulgarian Academy of Sciences, Natural Sciences, Ecology and Environmental Science – N 012).

The dissertation shall be defended at a public meeting of the Dissertation Defense Panel at 1 p.m. on 6th May 2026 in Conference Room 101 of the State Scientific Research Institute Nature Research Centre.

Address: Akademijos str. 2 LT-08412, Vilnius, Lithuania
Tel. +37052729257;
E-mail: sekretoriatas@gamtc.lt

https://doi.org/10.15388/vu.thesis.913
https://orcid.org/0000-0002-2015-8679

VILNIAUS UNIVERSITETAS
VALSTYBINIS MOKSLINIŲ TYRIMŲ INSTITUTAS GAMTOS TYRIMŲ CENTRAS

Gabrielė Bumbulytė-Žukevičienė

Cheminių signalų įtaka *Tenebrio molitor* elgsenai

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Mokslinis vadovas – prof. habil. dr. Vincas Būda (Valstybinis mokslinių tyrimų institutas Gamtos tyrimų centras, gamtos mokslai, ekologija ir aplinkotyra – N 012).

Gynimo taryba:
Pirmininkas:
Prof. Dr. Algimantas Paulauskas (Vytauto Didžiojo universitetas, gamtos mokslai, ekologija ir aplinkotyra – N 012).

Nariai:
prof. habil. dr. Eugenija Kupčinskienė, (Vytauto Didžiojo universitetas, gamtos mokslai, botanika – N 013),
dr. Rasa Bernotienė (Valstybinis mokslinių tyrimų institutas Gamtos tyrimų centras, gamtos mokslai, ekologija ir aplinkotyra – N 012),
dr. Irena Nedveckytė (Vilniaus universitetas, gamtos mokslai, ekologija ir aplinkotyra – N 012),
dr. Teodora Toshova (Bulgarijos mokslų akademijos Biologinės įvairovės tyrimų institutas, gamtos mokslai, ekologija ir aplinkotyra – N 012).

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Adresas: Akademijos g. 2, LT-08412, Vilnius, Lietuva.
Tel. +37052729257;
El. paštas: sekretoriatas@gamtc.lt

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ABBREVIATIONS

EO – essential oil
VOC – volatile organic compound
PDA – potato dextrose agar

LIST OF PUBLICATIONS ON THE DISSERTATION TOPIC

The dissertation is based on the following co-authored publications in journals with an impact factor, as referenced in the Clarivate Analytics Web of Science database. Throughout the text, the publications are referred to using the Roman numerals:

I. **Bumbulytė, G**.; Būdienė, J.; Būda, V., 2023. Essential Oils and Their Components Control Behavior of Yellow Mealworm (*Tenebrio molitor*) Larvae. *Insects*, *14*(7), p.636. DOI: 10.3390/insects14070636. (Q1, IF 2.7).

II. **Bumbulytė, G**., Auškalnis, A., Būda, V., 2025. Seed fatty acids modify oviposition of *Tenebrio molitor* (Coleoptera: Tenebrionidae). *Plants*, *14*(6), p.848. DOI: 10.3390/plants14060848. (Q1, IF 4.1).

III. Skrodenytė-Arbačiauskienė V., **Bumbulytė, G.**, Auškalnis, A., Būda, V., 2026. Gut microbiota and frass volatiles: behavioral responses of *Tenebrio molitor* and *Tenebrio obscurus*. *Journal of Pest Science, 99*(53). DOI: 10.1007/s10340-025-02012-y (Q1, IF 4.1).

IV. **Bumbulytė G.**, Čepukoit D., Būdienė J., Burokienė D., Būda V., 2026. Yellow mealworm infestation caused by *Hyphopichia burtonii* (Saccharomycetales: Pichiaceae) and associated changes in VOC. *Journal of Invertebrate Pathology*, *216* p.108568. DOI: 10.1016/j.jip.2026.108568 (Q1, IF 2.4).

V. **Bumbulytė G.**, Lučinskaitė I., Čepukoit D., Būda V., 2026. Search for *Tenebrio molitor* behaviorally active compounds in *Aspergillus flavus*-infected grain. *Journal of Economic Entomology* (submitted for publication).

AUTHOR CONTRIBUTIONS IN THE CORRESPONDING PAPERS

All authors have read and approved the published version of the manuscripts.

I. Conceptualization V.B.; formal analysis and investigation **G.B**.; methodology **G.B**. and J.B.; writing – original draft **G.B**. and J.B; writing-review and editing V.B.

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IV. Conceptualization V.B.; formal analysis and investigation **G.B**., J.B and D.Č.; methodology V.B., **G.B**., D.Č. and D.B; writing – original draft **G.B**.; writing-review and editing V.B.

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LIST OF CONFERENCE PRESENTATIONS ON THE SUBJECT OF THE DISSERTATION

1. **Bumbulytė, G**., Būda, V. (2022-08-08 - 12). Effects of essential oils and their compounds on mealworm (*Tenebrio molitor* L.) larvae. 3rd Joint Meeting of the International Society of Chemical Ecology (ISCE) and the Asia-Pacific Association of Chemical Ecologists (APACE): abstract book p. 217, Kuala Lumpur, Malaysia (poster presentation),
2. **Bumbulytė, G**., Būda, V. (2023-04-25). Influence of different substrates on oviposition of *Tenebrio molitor*. International Conference of Life Sciences, The COINS: abstract book, p. 80, Vilnius, Lithuania (poster presentation),
3. **Bumbulytė, G.** (2023-05-17). Insects – food and feed of the future. Republican Scientific-Practical Conference Modern Technologies for Sustainable Environment 2023. Vilnius, Lithuania (oral presentation),
4. **Bumbulytė, G.**, Būda, V. (2023-07-23/27). Towards chemical ecology of *Tenebrio molitor* larvae and adults. 38th Annual Meeting of the International Society of Chemical Ecology: abstract book p. 173, Bengaluru, India (poster presentation),
5. **Bumbulytė, G**., Būda, V. (2024-07-14/18). Fungus infected grain: Reaction in males and females of yellow mealworm, *Tenebrio molitor* L. (Coleoptera, Tenebrionidae). 39th Annual Meeting of the International Society of Chemical Ecology: abstract book p. 173, Prague, Czechia (poster presentation),
6. **Bumbulytė-Žukevičienė**, G., Lučinskaitė, I., Čepukoit, D., Būda, V. (2024-11-26). Wheat grain infected by *Aspergillus flavus* modifies behavior of yellow mealworm, *Tenebrio molitor* L. The 13th International Conference Young Scientists For Advance Of Agriculture AGRISCI2024: abstract book p. 56, Vilnius, Lithuania (oral presentation),
7. **Bumbulytė-Žukevičienė** G., Auškalnis A., Būda V. (2025 m. balandžio 17 d.) Seed fatty acids modify oviposition of *Tenebrio molitor* (Coleoptera: Tenebrionidae). II scientific conference Food for life: abstract book, p. 81, Kyiv, Ukraine (oral presentation),
8. **Žukevičienė G.**, Skrodenytė-Arbačiauskienė V., Auškalnis, A., Būda, V. (2025-08-10 – 2025-08-15). Chemical Conversations in the Dark: VOCs and Microbiota Shape Behavior in *Tenebrio molitor* and *T. obscurus*. 110th annual Ecological Society of America meeting, Baltimore, Maryland, United States of America (poster presentation).

AWARDS

1. Research Council of Lithuania. Support to attend the 37th annual International Society of Chemical Ecology meeting in Malaysia (2022, award no. P-DAK-22-89),
2. In the International Conference of Life Sciences, the COINS Bumbulytė G. was awarded the most entrepreneurial poster by the Research Council of Lithuania. Vilnius, Lithuania (2023),
3. International Society of Chemical Ecology Student Travel Award. Prague, Czechia (2024),
4. Diploma for the best presentation in the 13th International Conference Young Scientists for Advance of Agriculture AGRISCI2024. Kaunas, Lithuania (2024),
5. Research Council of Lithuania. Scholarship for academic achievements (2024, award no. P-DAP-24-98),
6. Research Council of Lithuania. Support to attend the 39th annual International Society of Chemical Ecology meeting in Czechia (2025, award no. P-DAK-24-51),
7. Innovative Research Award at the International Food for Life Conference. Kyiv, Ukraine (2025).

SCIENTIFIC PROBLEM

Tenebrio molitor L. (Coleoptera: Tenebrionidae), commonly known as the yellow mealworm, is closely associated with humans due to its dual role as an agricultural pest and a widely used food source. Traditionally, it is a major pest of stored products (especially grains and flour), causing quantitative and qualitative losses through feeding and contamination. The latter compromises food safety by introducing allergenic particles and promoting microbial growth (Deutsch et al., 2018; Villa et al., 2023). Simultaneously, *T. molitor* is increasingly cultivated as a sustainable protein source for the food and feed industries. This pest-beneficial insect paradox underscores the need for knowledge of pest control in storage environments and for optimizing growth and reproduction in farming systems, highlighting the need for chemical ecology data to design targeted strategies for both contexts.

Historically, pest management has relied on synthetic insecticides and fumigants (Rajashekar et al., 2010). However, environmental concerns, health risks, and insecticide resistance demand ecologically sound alternatives. One promising approach uses plant-derived compounds, such as essential oils (EOs), which exhibit repellent (Nerio et al., 2010), antifeedant (Ebadollahi, 2011), and insecticidal (Ebadollahi & Jalali Sendi, 2015) properties. Yet EO efficacy is species- and stage-specific, and studies remain limited to other stored-product coleopteran pests, such as *Tribolium castaneum* (Herbst) (Olivero-Verbel et al., 2013). Behavioral responses of *T. molitor*, especially larvae, remain poorly characterized.

Fungus-infected stored grain may provide additional natural repellents beyond plant-derived EOs. Because moldy grain can impair or even poison *T. molitor* larvae (Reiss, 1973), insects likely benefit from detecting and avoiding infected substrates at a distance through fungal volatile organic compound (VOC) cues. Similar long-range recognition of mold-infected substrates has been shown in *Plodia interpunctella* (Hübner), and shared VOCs involved in this process have been identified (Būda et al., 2016). Furthermore, many assays lack chemical identification and dose-response data, limiting practical applications in pest control and behavioral modulation during rearing.

Seed-insect interactions are another underexplored chemoecological aspect. Seeds produce diverse chemical cues that may influence oviposition. Although plant VOCs mediate herbivory above and below ground (Johnson & Rasmann, 2015), seed-specific chemoecological mechanisms remain largely unknown. This gap limits our understanding of host selection and hinders optimization of substrates for industrial breeding. Identifying seed compounds that deter or attract egg-laying females could simultaneously improve pest deterrence and enhance reproductive efficiency.

Microbial organisms make it more complex the ecology of *T. molitor*. In mass-rearing and storage environments, insects encounter a wide range of fungi and yeasts from grain substrates (Wakil et al., 2014; Cortes-Ortiz et al., 2016). Although entomopathogenic fungi such as *Metarhizium anisopliae* (Metchnikoff) and *Beauveria bassiana* (Bals.-Criv.) Vuill. are well known for causing disease outbreaks in industrial insect production (Araújo & Hughes, 2016; Hong et al., 2024), yeasts have historically received far less attention as potential pathogens. Overall, insect-yeast interactions are a complex and still insufficiently understood component of insect ecology.

Investigating frass-mediated communication and the influence of the microbiota represents an emerging frontier. Frass (a by-product of insect breeding, consisting of leftover substrate, including spent feedstock, insect feces, and cuticles) serves as a microhabitat for microorganisms that affect host fitness, physiology, and ecology (Magura et al., 2024). Insect gut microbiota contribute to nutrient metabolism, detoxification, pathogen resistance, and reproduction (Engel & Moran, 2013; Yang et al., 2021a, b; Wang et al., 2024a, b, 2025; Ding et al., 2024; Yasika & Shivakumar, 2025) and can even serve as a source of semiochemicals such as pheromones and kairomones (Dillon et al., 2002; Sharon et al., 2011; Leroy et al., 2011a, b; Zhou et al., 2025). Frass-derived VOCs have been shown to mediate aggregation and mate attraction in certain insect species (Davis et al., 2013); however, their role in sex-specific communication remains poorly understood. Comparative analysis of the gut microbiota of *T. molitor* has so far been conducted only at the larval stage (Yang et al., 2021b). Therefore, it remains unclear whether the same differences persist in adult beetles and whether they differ between males and females. Moreover, studies directly linking microbiota profiles to behavioral assays are still lacking. Such work would help determine whether microbiota contributes to the production of behavior-influencing chemical cues and to potential behavioral differences.

Tenebrio molitor behavior may be modulated by chemical cues from plant-derived materials, fungi, and the insect's frass. However, the identity of the key cues and the magnitude of their effects on orientation, feeding, and oviposition remain poorly resolved. Clarifying these relationships could support both targeted pest-control strategies and improved performance in industrial insect-rearing systems.

OBJECTIVE AND MAIN TASKS OF THE STUDY

The objective was to identify environmentally friendly compounds that determine certain behavioral features of larvae and adults of the yellow mealworm (*Tenebrio molitor* L.).

The following tasks were set to achieve the objective:

1. To evaluate the effects of essential oils and fungus-infected grain, as natural mixtures of volatile organic compounds (VOCs), on *T. molitor* behavior.
2. To determine which single compounds, within selected natural VOC mixtures showing attractive or repellent effects on *T. molitor*, elicit behavioral responses.
3. To determine whether VOCs emitted by stored grains affect oviposition behavior in *T. molitor* females and, if an effect is detected, to isolate and identify the active compounds.
4. To evaluate whether VOCs from *T. molitor* frass act as semiochemical signals mediating behavioral responses among conspecific beetles.
5. To identify the causative agent(s) of infestation detected in a laboratory *T. molitor* culture and to assess whether infestation alters the composition of emitted VOCs and insect behavior.

STATEMENTS TO BE DEFENDED

1. *Tenebrio molitor* larvae are repelled by volatile organic compounds (VOCs) from essential oils, as well as by individual constituents of these oils.
2. Adult *T. molitor* beetles detect grains infected with *Aspergillus flavus* and avoid infestation.
3. Fatty acids (oleic, palmitic, linoleic, and stearic) significantly inhibit oviposition in *T. molitor* females.
4. VOCs emitted from *T. molitor* frass serve as semiochemical signals in intraspecific interactions.
5. The composition of the gut microbiota in *T. molitor* is sex-specific.
6. *Tenebrio molitor* can be infested by the yeast *Hyphopichia burtonii*, this yeast reduces the production of reproduction-related pheromones and alters the insect's VOC profile, while increased limonene levels serve as a suitable marker of infestation.

NOVELTY AND THE RELEVANCE OF THE RESEARCH

1. For the first time, naturally derived agents that influence *Tenebrio molitor* behavior were identified: five essential oils with repellent activity and eight single behavior-active volatile organic compounds (VOCs) isolated from complex VOC mixtures, providing a basis for behavioral manipulation and a sustainable alternative to insecticides.
2. For the first time, *T. molitor* beetles were shown to detect VOCs emitted by *Aspergillus flavus*-infected grains and to avoid them.
3. For the first time, fatty acids were shown to affect oviposition; these new data support optimizing substrates for industrial rearing of *T. molitor*.
4. For the first time, VOCs emitted from *T. molitor* frass were shown to function as intraspecific chemical signals, opening new strategies for population regulation and behavioral control.
5. For the first time, significant sex-specific differences were observed in the gut microbiota of adult *T. molitor* beetles.
6. For the first time, infestation by the yeast *Hyphopichia burtonii* was detected in *T. molitor* colonies.
7. For the first time, *H. burtonii* infestation was shown to alter the VOC profile of *T. molitor* by increasing limonene levels, thereby enabling early diagnostic monitoring of microbiological contamination and decreasing sex pheromone production.

1. BRIEF LITERATURE REVIEW

The yellow mealworm, *Tenebrio molitor* L. (Tenebrionidae), belongs to a species-rich and ecologically diverse family with more than 18,000 described species worldwide, in Lithuania's temperate climate, only a few *Tenebrio* species occur (Pileckis, 1976; Tamutis et al., 2011). Adults are typically dark, with a broadened anterior head, and species occupy habitats ranging from soil (feeding on and damaging roots) to synanthropic environments such as houses, barns, and warehouses, where they exploit grain, flour, and other stored products (Lešinskas & Pileckis, 1967). Native to Europe but now cosmopolitan, *T. molitor* associates closely with storage environments and grocery outlets; while present, its populations are often relatively small and not among the most destructive storage pests (Ramos-Elorduy et al., 2002), a status that nevertheless demands targeted knowledge for suppression as a pest and optimization as a farmed resource.

Morphology and life history define the ecological plasticity of *T. molitor*. Adults measure 1.25-1.8 cm, with a glossy black dorsum, brown ventrum, and brown antennae and legs; the head is free (not covered by the pronotum), the pronotum is transverse and narrower than the elytra, with sharp lateral margins and right-angled hind corners, and the elytra show longitudinal punctate grooves; hind wings are fully developed, the scutellum is a transverse pentagon, and the first hind tarsal segment is nearly as long as the second and third combined (Lešinskas & Pileckis, 1967). Fecundity is high: post-mating, females lay ~500 eggs (1.6-1.8 mm) singly or in clusters in food or in crevices of walls, boxes, and floors, and eggs hatch in 4-19 days; larvae (to ~30 mm) shift from white to yellow-brown, carry two upturned terminal spines, and molt repeatedly while feeding on plant materials and grains (Hill, 2002). Pupae (16-17 mm) are whitish, later brownish, with two terminal spines; pupation lasts 3-30 days depending on conditions. Adults emerge from June through autumn, live ~70 days on average, and reproduce during warmer months; overwintering commonly occurs in the larval stage (Pileckis & Žuklys, 1974; Pileckis, 1976). These traits frame opportunities for both pest suppression and controlled rearing.

Chemical ecology mediates mate choice and population quality in dense, synanthropic settings. Sex-specific pheromones include 4-methyl-1-nonanol released by females to attract males (Tanaka et al., 1986) and (*Z*)-3-dodecenyl acetate emitted by males to attract females (Bryning et al., 2005). Signal quality is dynamic: inbreeding reduces male pheromone attractiveness (Pölkki et al., 2012), and females preferentially orient toward cues from less-related males over close kin (Bernstein et al., 1987), indicating chemically mediated mate choice that can modulate gene flow.

Diet and rearing conditions anchor plant-insect interactions and scale behavioral responses. In nature, mealworms exploit plant materials and animal-origin substrates (meat, feathers) (Ramos-Elorduy et al., 2002). In laboratory and industrial systems, they are typically maintained on bran or cereal flours (wheat, oat, corn) with fruits or vegetables to provide moisture and supplemental protein (soybean meal, skim milk powder, yeast) (Aguilar-Miranda et al., 2002). Diets near ~20% protein with at least ten essential amino acids (leucine, isoleucine, valine, threonine, lysine, arginine, histidine, methionine, phenylalanine, tryptophan) support growth and reproduction (Ramos-Elorduy et al., 2002; Lundgren, 2009). *Tenebrio molitor* tolerates broad thermal ranges, feeding optimally between 15-40 °C and surviving stress from 0-15 °C and 40-45 °C (Li et al., 2013), parameters that define resilience in warehouses and operating envelopes for farming.

Interest has grown due to high feed-conversion efficiency, rapid development, and versatility as a protein source for food, feed, and bioconversion (Oonincx et al., 2015; Villa et al., 2023). Larvae contain ~45-60% crude protein and 25-35% lipids (dry matter), with essential amino acid profiles comparable to soybean meal. Substrate composition strongly influences growth rate, feed conversion, and body composition, underscoring the importance of diet formulation for yield and nutrient quality (Oonincx et al., 2015). These dependencies imply that chemical cues associated with substrates, including plant VOCs and microbial metabolites, shape behavior and can scale to system performance.

Electrophysiological and behavioral assays show that larvae and adults detect and respond to VOCs from prospective food sources; aldehydes such as hexanal and nonanal elicit strong antennal responses and guide aggregation and substrate selection, informing rearing design and automated sorting (Villa et al., 2023). In parallel, tenebrionid interactions with plant-derived compounds (e.g., essential oils) and seed odors represent core plant-insect interaction axes that can modulate oviposition and feeding, areas that are still underdeveloped for *T. molitor* relative to other stored-product beetles.

Microbe-host interactions add an interspecific dimension to chemical ecology. *T. molitor* mounts robust innate immune responses, and diet-driven shifts correlate with differences in larval growth (Yang et al., 2021b). Because diet alters microbial metabolites and host immunity, it likely modulates chemosensory processing and behavior, creating feedback among food, microbes, and chemical communication that extends to frass-associated VOCs and social signals in intra- and interspecific contexts.

Across these domains, the main picture is clear: *T. molitor* thrives at the interface of chemical ecology (pheromones and food-associated VOCs), nutritional physiology (protein and essential amino acids), and microbe-host interactions (diet-shaped microbiota and immunity). Yet key gaps persist in

oviposition-modulating cues (stimulants and deterrents), environmentally friendly repellents tailored to life stages, and the behavioral roles of VOCs emitted from frass, particularly in intraspecific scenarios. Furthermore, the ways in which diet and microbiota reshape chemical signaling and behavioral outcomes remain incompletely understood. Addressing these gaps will support knowledge-based suppression in storage and optimization in farming, two applications served by the same chemical-ecology toolkit.

2. MATERIALS AND METHODS

A detailed description of the collected material and the methods applied in the present dissertation is provided in the published papers and in one paper currently in press. A brief outline, along with corresponding references, is provided below.

2.1 Insects

Larvae of *Tenebrio molitor* were obtained from laboratory cultures maintained at the Laboratory of Chemical Ecology and Behavior, State Scientific Research Institute Nature Research Centre, Vilnius, Lithuania, since 2019. Rearing diets varied slightly across experiments:

Oat flakes (70%), wheat bran (20%), dry yeast (10%), with carrot slices (**Paper I**). Wheat bran with carrot pieces (**Paper II**). Wheat bran (90%) plus dry yeast (10%), with carrot slices (**Papers III-V**). In some experiments, yeast was used as a nutritional supplement to enrich the diet of *Tenebrio* beetles.

Environmental conditions were maintained at 25-26 °C, 60±10% relative humidity, and a 12:12 h light-dark cycle (**Papers I-V**). Medium-sized larvae (14-20 mm) were used for larval behavioral tests (**Paper I**). Sexually mature beetles aged 7-15 days were used for adult behavioral and oviposition tests (**Papers II-V**). Pupae were sexed based on terminal segment morphology to obtain virgin adults (**Papers II-V**).

In **Paper III**, for gut microbiota analysis, 40 randomly selected 10-15-day-old adults were euthanized at −20 °C for ≥1 h, surface-sterilized with 75% ethanol, and dissected immediately. Each sample consisted of the dissected intestines of five individuals, along with their digested/undigested contents; samples from males and females were prepared separately. Intestinal samples were collected in "DNA/RNA Shield Lysis Tubes (Microbe)" and stored until further analysis.

2.2 Biologically Active Compounds and Substrates

2.2.1 Complex Volatile Mixtures

Six essential oils (the cheapest and most readily commercially available)– thyme (*Thymus vulgaris* L.), eucalyptus (*Eucalyptus globulus* Labill.), spearmint (*Mentha spicata* L.), lavandin (*Lavandula × hybrida* Rev.), East-Indian lemongrass (*Cymbopogon flexuosus* (Nees ex Steud.) W.Watson), and clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry) – were purchased from commercial suppliers (**Paper I**).

An *Aspergillus flavus* culture was grown on potato dextrose agar (PDA) at 25 °C for 7 days, until sporulation (**Paper V**). Media and grain samples were

sterilized by autoclaving (125 °C, 1.2 atm), and all manipulations were performed aseptically in a Class II laminar flow cabinet to prevent cross-contamination. Sterile grain portions (2 g in 7 mL vials) were inoculated with spores collected using a sterile loop from fully sporulated 7-day-old PDA plates; spores were evenly distributed by adding 300 µL of sterile water and maintaining moisture. Inoculated grains were incubated for 0, 5, 10, and 20 days at 27 °C in a climatic chamber.

2.2.2 Analytical Standards

Analytical standards included: (*R*)-(−)-carvone, limonene, myrcene, *γ*-terpinene, *cis*-sabinene hydrate, terpinene-4-ol (**Paper I**), oleic, linoleic, palmitic, and stearic acids (**Paper II**), 1-octen-3-ol, hexanal, 3-methylbutanol, 1-pentanol, toluene, 1-hexanol, and 2-methylfuran (**Paper V**).

2.2.3 Yeast

In **Paper IV**, substrates were also used to cultivate and maintain *Hyphopichia burtonii* isolates obtained from infected *T. molitor*. Two standard media were used for fungal isolation and growth:

- PDA,
- Malt Extract Agar.

In addition, agar-based substrates were prepared to expose *T. molitor* larvae to infectious agents during mortality assays (**Paper IV**): sterile agar served as a control substrate, while agar inoculated with sporulating *H. burtonii* or *Metarhizium anisopliae* provided standardized infection treatments.

Substrates used in behavioral assays in **Paper IV** included small containers with either healthy larvae or *H. burtonii*-infected larvae, enabling discrimination tests without introducing additional chemical substrates.

2.2.4 Substrates for Oviposition

Substrates for oviposition and feeding assays included ground and sieved oat flakes (1 mm), wheat flour, rapeseed flour, and wheat bran (**Paper II**). Quartz sand (0.0-0.4 mm) was washed and heated at 150 °C for 2 h before use (**Paper II**).

Fatty acids tested were prepared in hexane and applied to substrates at concentrations of 0.05%, 0.5%, and 5% (**Paper II**).

2.3 Experimental Design

2.3.1 Attraction and Repellency Bioassays

Two-zone choice arena: Petri dish (10.5 cm) lined with filter paper; one side treated with EO or compound, the other with solvent control (**Papers I and V**). Stimuli were applied as five 2 µL spots (total 10 µL). Larvae or adults were introduced at the center; movement was recorded for 5 min using EthoVision XT 12 (**Papers I and V**).

Pit-fall two-choice test: Petri dish with two tubes leading to stimulus or control chambers (**Papers III-V**). Used to test yeast and fungal VOCs (**Papers IV and V**) and frass cues (**Paper III**).

2.3.2 Oviposition Bioassays

Mesh-bottomed boxes with Petri dishes containing 20 g of substrate beneath the mesh. Ten males and ten females (intact or antennae-less) were introduced, with a piece of carrot as a water source. After 24 h, eggs were counted. Variants included (**Paper II**):

a. Substrate preference: oat, wheat, rapeseed flour, wheat bran,
b. Sand substrate: sand vs. wheat flour,
c. Oat extract: hexane extract of oats applied to sand,
d. Oat extract fractions: separated fractions applied to extracted oat flour,
e. Fatty acid treatments: sand treated with fatty acids at three concentrations.

2.3.3 Mortality Test

A mortality assay was conducted to compare the pathogenic effects of *H. burtonii* and the entomopathogenic fungus *M. anisopliae* on *T. molitor* larvae (**Paper IV**).

Three treatment groups (n = 25 larvae each; size 1.2-1.4 cm):

- *M. anisopliae*-infected,
- *H. burtonii*-infected,
- Control (sterile agar).

A 200 µL aliquot of spore suspension (1×10^7 spores/mL) was applied onto a 0.5 cm agar piece used as the infection substrate (**Paper IV**).

Larvae were fed inoculated agar for 3 days, after which 0.5 g of sterile wheat bran was added. Survival was monitored daily for 30 days; individuals were considered dead if unresponsive to tactile stimulation (**Paper IV**).

2.4 GC-MS Analysis and Compound Identification

VOC composition was analyzed using a Shimadzu GC/MS-Q2010 PLUS with an Rxi-5 Sil MS column, temperature-programmed analysis, and helium as the carrier gas (**Papers I, III, IV**). Compounds were identified using retention indices and spectral library matching (≥ 90% match).

2.5 Isolation and Morphological Identification of Yeast

Tenebrio molitor beetles infected with yeast were dissected with a sterile scalpel, and a small fragment of the fungal mass was extracted with sterile forceps. The sample was pressed into the media (**Paper IV**).

Morphological identification of 7-day-old colonies was performed using a Nikon Eclipse Ci microscope, with hyphal structure and spore morphology examined (**Paper IV**).

2.6 Molecular Analyses of Microbiota and Yeast Isolates

Gut samples were submitted to the ZymoBIOMICS Targeted Sequencing Service (Zymo Research, Irvine, CA) for 16S rRNA gene V3-V4 region amplicon sequencing and bioinformatics analysis (**Paper III**). Microbial DNA was extracted using the ZymoBIOMICS-96 MagBead DNA Kit with blank and mock-community controls, and libraries were prepared with the Quick-16S™ Plus NGS Library Prep Kit. Sequencing was performed on an Illumina NextSeq (600 cycles; 30% PhiX). ASVs were inferred using DADA2; taxonomy, diversity analyses, and differential taxa testing (LEfSe) were performed in QIIME v1.9.1 using the curated Zymo Research 16S database, and differential taxa testing was performed using LEfSe with linear discriminant analysis (LDA) (**Paper III**).

Yeast's genomic DNA was isolated using a modified CTAB protocol. ITS and large subunit ribosomal DNA (LSU) sequences were assembled in Geneious Prime (2025.1.2), identified using BLASTN, aligned with representative type strains, and analyzed using the maximum likelihood method in MEGA12 with 1000 bootstrap replicates. Resulting sequences were deposited in GenBank (**Paper IV**).

2.7 Statistical analysis

Data processing was performed in Microsoft Excel, and statistical analyses were conducted in STATISTICA and R.

Behavioral assays: Wilcoxon matched-pairs test (**Papers I and V**), exact binomial test (**Papers III and V**).

Oviposition assays: One-way ANOVA with Tukey's HSD, Friedman test with Wilcoxon signed-rank post hoc (Bonferroni correction), Mann-Whitney U test (**Paper II**).

Microbiota diversity: ANOVA, PERMANOVA, PCoA (**Paper III**).

Survival analysis: survival data were analyzed using Kaplan-Meier survival curves. Differences among treatments were evaluated using the log-rank (Mantel-Cox) test, with χ^2 statistics and corresponding *p*-values reported. These analyses were conducted in R using the *Survival* and *Survminer* packages (**Paper IV**).

Significance threshold: $p < 0.05$ for all tests.

3. RESULTS AND DISCUSSION

In-depth descriptions of the results and discussions of the data were published in papers that form the foundation of this thesis (**Papers I-IV**), including one paper currently in press (**Paper V**). Below are summaries of the dissertation results corresponding to each task of the study.

3.1 *Tenebrio molitor* Response to VOC

3.1.1 Essential Oils

When exposed to essential oils (EOs), larvae spent significantly less time in EO-treated zones for lemongrass, thyme, lavandin, clove, and spearmint, whereas eucalyptus showed no significant effect (**Paper I**). Across oils, behavioral suppression ranged from 1.84 times (eucalyptus) to 5.74 times (spearmint) relative to the control, based on combined time and distance measures (**Paper I**). Distance moved within EO zones also decreased: 2.23 times shorter with eucalyptus, 5.15 times with lavandin, 5.29 times with thyme, 6.47 times with lemongrass, 12.76 times with clove, and 17.24 times with spearmint, relative to the paired control zone (**Paper I**). Exposure to lemongrass EO elicited observable escape behaviors, including falling to the side, cringing, and curling (**Paper I**). Using combined time and distance criteria, EOs were categorized as weak (eucalyptus, lemongrass, thyme, lavandin), moderate (clove), and strong (spearmint) repellents (**Paper I**). The graded repellency across EOs aligns with their chemotypes and the dominance of specific monoterpenes: carvone-rich spearmint and eugenol-rich clove produced the strongest behavioral suppression, consistent with prior reports of EO bioactivity against stored-product beetles (George et al., 2009; Martínez et al., 2018).

Gas chromatographic profiling revealed heterogeneous chemical compositions across EOs, and only three VOCs – limonene, linalool, and *trans*-caryophyllene – were shared among all six EOs, often at highly variable abundances (**Paper I**).

Single-compound assays identified terpinene-4-ol as the most potent repellent, significantly reducing distance moved at all tested doses (0.01, 0.1, 1 mM) (**Paper I**). Carvone suppressed locomotion dose-dependently (distance 1.76 times shorter at 0.1 mM and 5.02 times shorter at 1 mM), with significant time avoidance emerging at 1 mM (3.62 times) (**Paper I**). *cis*-Sabinene hydrate reduced distance at 1 mM (control/stimulus ≈ 1.92), whereas limonene, myrcene, and *γ*-terpinene did not elicit significant changes across 0.01-1 mM (**Paper I**). Collectively, these results identify terpinene-4-ol and carvone as key bioactive compounds underpinning the strong repellency of specific EOs to larvae. This suggests that minor constituents can modulate

potency via mixture interactions (antagonism or masking), a plausible explanation for the weaker behavioral output of thyme EO despite containing terpinen-4-ol (Satyal et al., 2016; **Paper I**).

3.1.2 Frass

Two-choice olfactometer assays demonstrated pronounced sex-dependent responses to frass VOCs (**Paper III**). *Tenebrio molitor* males were significantly attracted to frass from both males and females, consistent with frass semiochemicals mediating aggregation-like responses and, potentially, sex-related attraction. However, the specific active compounds and their functional roles remain to be identified. *T. molitor* females exhibited selective attraction only to male frass, consistent with a putative role in mate recognition or courtship (**Paper III**). These data highlight frass VOCs as ecologically relevant signals that can structure intraspecific communication and potentially mediate interspecific interactions in Tenebrionidae (**Paper III**).

Historic evidence that *T. molitor* larvae aggregate around frass via contact cues, together with our adult olfactory assays using frass volatiles, suggests life-stage-specific sensory pathways, contact-mediated responses in larvae and volatile-mediated responses in adults, that could be exploited for monitoring and, potentially, for mating disruption (Weaver et al., 1989; Paper III). The observed attraction to frass volatiles indicates that frass VOCs function as semiochemicals in adult interactions. More broadly, microorganisms are known to modulate beetle-associated VOC emissions in other systems, suggesting that microbial communities could plausibly contribute to the frass volatile profile observed here (Davis et al., 2013; Silva-Junior et al., 2018; Sánchez-Cruz et al., 2020).

3.1.3 Substrate-Associated Fungi

Adult *T. molitor* consistently preferred uninfected wheat grain over grain infected with *Aspergillus flavus*. Females avoided infected grain immediately after inoculation (day 0), whereas males developed avoidance by day 5 and maintained it thereafter (**Paper V**). Moldy grain can negatively affect the development of *T. molitor* larvae and even be toxic to them (Reiss, 1973).

To clarify the chemical basis of adult *T. molitor* avoidance of *Aspergillus flavus*-infected wheat grain, a literature review of VOCs associated with fungus-infected wheat was conducted. On this basis, seven compounds were selected for behavioral testing, as they had been repeatedly identified in the VOC profile of *A. flavus*-infected wheat in at least three published studies.

Sex-specific VOC effects were resolved. 1-Octen-3-ol was strongly repellent to males at the lowest concentration tested (1 mM), while it neither

repelled nor attracted females across 1-100 mM (**Paper V**). Hexanal repelled males at ≥ 10 mM and females at all tested concentrations (1-100 mM) (**Paper V**). 1-Hexanol repelled males at the highest dose and attracted females at lower doses, with the attractive effect diminishing at the highest concentration (**Paper V**). Hexanal, abundant in unripe grain and declining upon maturation, likely acts as a freshness/unsuitability cue that increases with fungal activity, providing a mechanistic link between grain status and beetle avoidance (Būda et al., 2016; Ji et al., 2017). The bidirectional effect of 1-hexanol (female attraction vs. male repellency) reveals potential for sex-targeted monitoring tools and indicates that the net behavioral output depends on blend composition in the fungus-grain headspace (**Paper V**).

3-Methylbutanol elicited attraction in females at a single concentration tested but did not repel males; 1-pentanol, toluene, and 2-methylfuran were behaviorally neutral within the dose range (**Paper V**).

3.2 *Tenebrio molitor* Female Oviposition

3.2.1 Different Substrates

Tenebrio molitor females with intact antennae laid significantly more eggs on oat and wheat flour than on rapeseed flour or wheat bran (ANOVA $F(56.02) = 3$, $p < 0.001$), indicating these cereal flours as preferred oviposition substrates (**Paper II**). Removal of antennae (attenuating antennal olfaction) did not abolish substrate preferences but increased variance. Oviposition remained lowest on wheat bran, with oat and wheat flour still most suitable (ANOVA $F(5.601) = 3$, $p = 0.0229$) (**Paper II**). To isolate chemical effects from physical substrate properties, sand (inorganic, SiO2-based) was validated as a neutral oviposition matrix supporting ~78% of egg numbers relative to wheat flour; the ~22% reduction was statistically significant, yet sand remained fit-for-purpose for chemical cue assays (ANOVA $F(5.203) = 3$, $p = 0.0236$) (**Paper II**).

Introducing oat extract into sand strongly inhibited oviposition: females deposited only ~4% of the eggs observed on pure sand, indicating a strong inhibitory effect of oat-derived chemical constituents (**Paper II**). This effect is most likely explained by the fact that, in intact oat flour, females respond to a complex combination of positive and negative chemical and physical cues, whereas in sand supplemented with oat extract only the extract-borne inhibitory cues remain, resulting in suppressed oviposition.

Fractionation trials further showed that both tested extract fractions reduced egg-laying when added to extracted oat flour, whereas extracted oat alone (depleted of soluble VOCs) supported higher oviposition; the effect was significant for females with intact antennae (Friedman $\chi^2(3) = 19.1$, $p < 0.001$)

but not for antenna-less females (χ^2 (3) = 7.4, p = 0.06018), implicating antennal olfaction in the perception of inhibitory cues (**Paper II**).

These data distinguish physical from chemical determinants of oviposition: even on nutritionally adequate substrates, chemical cues bias site choice, whereas short-range assays with antenna-less females indicate that distal olfaction is not obligatory at the deposition site (Gerber & Sabourin, 1984; Deruytter et al., 2019). The strong inhibition by oat extract and its fractions indicates multiple semiochemicals – including VOCs – that influence reproductive allocation; identifying these cues opens avenues for formulating oviposition-deterrent barriers for rearing or storage (**Paper II**; Tasin et al., 2011; Lei et al., 2024).

3.2.2 Fatty Acids

Guided by the prominent lipid layer in oat extract and published compositional data, four abundant fatty acids – linoleic, oleic, palmitic, and stearic – were evaluated in two-choice oviposition assays (**Paper II**). Across acids, 0.5% and 5% concentrations significantly suppressed total egg production in both treated and untreated options (ANOVA, $F(5, 68) = 12$, $p < 0.001$), indicating not only local avoidance but also an overall inhibitory (possibly stress-mediated) effect on oviposition (**Paper II**). Repellent properties were compound- and dose-dependent: significant choice asymmetries were observed at 5% for oleic and linoleic acids and at 0.5% for linoleic and palmitic acids, whereas 0.05% produced neither repellency nor inhibition (**Paper II**). These data position common cereal fatty acids as quantifiable modulators of reproductive allocation and site selection in *T. molitor* (**Paper II**).

Dose-dependent inhibition and repellency by common cereal fatty acids align with deterrence patterns reported in other insects and suggest a practical threshold (< 0.5% each) for oviposition media where reproduction is desirable (Roh et al., 2023; Klüber et al., 2024). Knowing the substrate's fat content (e.g., elevated oleic/linoleic in rapeseed, substantial linoleic in wheat/oat) can be valuable in industrial insect rearing to maximize the number of eggs in the shortest possible time (Kouřimská et al., 2018; Lidon et al., 2019; Chew, 2020).

3.3 *Tenebrio molitor* microorganisms

3.3.1 Gut Microbiota

Amplicon sequencing produced 4,671,839 high-quality, chimera-free reads from 16 gut samples, enabling robust analyses of diversity and composition (**Paper III**).

An analysis of the alpha and beta diversity of the gut microbiota of *T. molitor* revealed significant interspecific (intersexual) differences. The species richness and evenness (Shannon and Simpson indices) of microbial communities in the gut of *T. molitor* females were significantly higher ($p = 0.009$ and $p = 0.02$, respectively) than those of males. Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity distances and PERMANOVA (999 permutations) also identified significant differences in the gut microbiota between males and females within the *T. molitor* species ($p = 0.024$) (**Paper III**).

The relative abundance of the gut microbiota in *T. molitor* was also assessed in males and females across various taxonomic ranks. Bacteria from the genus *Spiroplasma* (phylum Mycoplasmatota – class Mollicutes – order Entomoplasmatales – family Spiroplasmataceae) were found only in gut samples from male *T. molitor*, where they made up 43.2%. Meanwhile, bacteria from the genus *Enterococcus* (phylum Bacillota – class Bacilli – order Lactobacillales – family Enterococcaceae) were predominant in females and accounted for 51.7% (**Paper III**). The male-biased enrichment of *Spiroplasma* suggests functional divergence that could influence the host odor profile and warrants integrative metagenomic and metabolomic analyses (Segata et al., 2011). More broadly, sex-linked microbiome structure mirrors patterns observed in other beetles and may feed back into chemical communication through microbially derived precursors and VOCs (Engel & Moran, 2013; Engl & Kaltenpoth, 2018; Wan et al., 2020).

3.3.2 Infestation

A laboratory colony of *T. molitor* exhibited a progressive microorganism-associated disorder affecting both larvae and adults, characterized by visible external colonization and reduced vitality (**Paper IV**). Early signs included white, fungus- or yeast-like growths concentrated near the mouthparts, leg joints, and intersegmental regions. These lesions expanded over time and were accompanied by sluggish behavior and reduced responsiveness. Some adults displayed restricted locomotion when the legs and thorax were heavily affected (**Paper IV**).

Isolation and identification work demonstrated that the infestation agent was the yeast *Hyphopichia burtonii*. Molecular analysis showed near-complete sequence identity to a reference strain (CBS 2352), and, together with morphological traits, supported assignment of the isolate to *H. burtonii* (**Paper IV**). In the context, this finding is notable because yeasts are comparatively underrepresented among well-characterized disease agents in mass-reared insects, despite the documented capacity of rearing environments

and grain-based substrates to facilitate opportunistic microbial proliferation (Davis, 2015; Bernardi et al., 2019; Slowik et al., 2023).

From a fitness-impact perspective, the infestation caused measurable impairment but did not exhibit the acute lethality typical of the entomopathogenic fungus *Metarhizium anisopliae*. In comparative exposure assays, no mortality occurred in control or yeast-infested larvae during the 7-day observation period, whereas larvae exposed to *M. anisopliae* experienced rapid, complete mortality by day 7, with survival curves differing significantly among treatments (**Paper IV**). This contrast indicates that *H. burtonii* infestations may primarily function as sublethal stressors (e.g., reduced locomotion and vitality), potentially influencing productivity and welfare in rearing systems without immediately triggering colony collapse (**Paper IV**).

Yeast-associated individuals exhibited a pronounced restructuring of the *T. molitor* VOC profile compared with healthy conspecifics (**Paper IV**): in larvae, the VOC blend was chemically richer under yeast association (23 vs 16 compounds above trace level) and shifted from a profile dominated by hexanoic acid and propanoate esters in healthy larvae to one dominated by limonene, *trans*-caryophyllene, and 2,4,6-trimethyl-pyridine in yeast-associated larvae, alongside reduced nonanal and heptanoic acid. Adults showed the same overall direction of change (13 vs 10 detectable compounds), with healthy beetles characterized by a high relative abundance of *cis*-3-dodecen-1-ol acetate, whereas yeast-associated adults exhibited a marked increase in limonene and reductions in several compounds, including *cis*-3-dodecen-1-ol acetate and tridecyl acetate. Reduced pheromone emissions were also observed in infected adult individuals (**Paper IV**).

Notably, the VOC profile of *H. burtonii* in pure culture was distinct from insect headspace: only 9 compounds exceeded trace levels, and emissions were dominated by alcohols (especially 3-methyl butanol, ethanol, and phenylethyl alcohol), none of which were detected in either healthy or yeast-associated insects (**Paper IV**). This disparity supports the interpretation that yeast-host interaction (and/or host-derived substrates) substantially modifies the VOCs phenotype expressed in vivo, consistent with prior observations that limonene is only trace in *H. burtonii* cultured on standard media (Pina et al., 2009; Xiao et al., 2016).

Behavioral assays indicated limited colony-level containment by social avoidance. Adult beetles showed no significant preference between infested and healthy larvae in a two-choice design, suggesting that altered odor cues do not elicit avoidance and may therefore permit undetected spread through routine contact (**Paper IV**).

CONCLUSIONS

1. The following mixtures of volatile organic compounds (VOCs) exhibited repellent activity against *Tenebrio molitor*:
    a. in larvae, the strongest repellency was observed for VOCs from spearmint (*Mentha spicata*) essential oil, a moderate effect for clove (*Syzygium aromaticum*) essential oil, and the weakest effects for VOCs from lemongrass (*Cymbopogon flexuosus*), thyme (*Thymus vulgaris*), and lavandin (*Lavandula × hybrida*) essential oils,
    b. in adults, repellency was observed for VOCs emitted by grains infected with *Aspergillus flavus*.
2. Within the VOC mixtures, eight single compounds were identified that affected the behavior of *T. molitor* adults and larvae:
    a. six with repellent properties (carvone, *cis*-sabinene hydrate, terpinen-4-ol, 1-octen-3-ol, hexanal, and 1-hexanol),
    b. one attractant (3-methylbutan-1-ol),
    c. one compound with sex-dependent effects (1-hexanol).

    Among these, the strongest repellency in adults was observed for hexanal (active in both sexes) and 1-octen-3-ol (active only in males), both at 0.05-5% concentrations. In larvae, the strongest repellent effects were observed for terpinen-4-ol (0.05-5%) and carvone (0.5-5%).
3. Oviposition in *T. molitor* females is inhibited by fatty acids in the substrate: palmitic and linolenic acids at concentrations ≥ 0.5%, and oleic acid at concentrations ≥ 5%.
4. VOCs emitted from *T. molitor* frass mediate intraspecific interactions: frass from males promotes aggregation by attracting males and females, whereas female frass attracts males only. Gut microbiota composition also differed by sex, with bacteria from genus *Spiroplasma* most abundant in males and *Enterococcus* in females.
5. The widespread yeast *Hyphopichia burtonii* can infect *T. molitor* cultures, reducing pheromone emission and altering VOC composition; in particular, limonene increases markedly, making it a suitable marker for early detection of infection.

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SANTRAUKA

MOKSLINĖ PROBLEMA

Didysis milčius (*Tenebrio molitor* L.; Coleoptera: Tenebrionidae) yra plačiai žinomas ir su žmogaus veikla glaudžiai susijęs vabzdys. Jis yra svarbus keliais aspektais. Nuo seno yra žinomas kaip sandėliuojamų produktų (ypač grūdų ir miltų) kenkėjas, sukeliantis tiek kiekybinius, tiek kokybinius nuostolius dėl produkcijos sugadinimo ir užteršimo. Produkto užteršimas vabzdžių atmatomis (ūkinių vabzdžių ekskrementų, šėrimo substrato, ūkinių vabzdžių kūno dalių ir negyvų kiaušinių mišinys (Komisijos reglamentas (ES) 2021/1925, 2021)) blogina maisto kokybę, gali sukelti alergines reakcijas ir sudaro palankias sąlygas mikroorganizmų augimui bei dauginimuisi (Deutsch ir kt., 2018; Villa ir kt., 2023). Tuo pat metu *T. molitor* vis plačiau auginamas kaip tvarus, aukštos kokybės baltymų šaltinis žmonių maistui ir gyvūnų pašarams. Šis dvilypis – kenkėjo ir naudingo vabzdžio – vaidmuo parodo, kad reikalingos žinios, leidžiančios sandėliavimo aplinkoje tikslingai valdyti kenkėjo elgseną, o pramoninėse auginimo sistemose – optimizuoti veisimą ir reprodukciją. Abiem atvejais cheminės ekologijos tyrimai yra svarbūs, nes leidžia kurti tikslingas, konkrečioms sąlygoms pritaikytas strategijas.

Istoriškai kenkėjų kontrolė daugiausia remiasi sintetiniais insekticidais (Rajashekar ir kt., 2010). Siekiant kurti aplinkai palankius ir tvarius sprendimus ateityje, dėl galimos rizikos žmogaus sveikatai ir insekticidams atsparių populiacijų formavimosi didėja ekologiškai pagrįstų alternatyvų poreikis. Viena perspektyviausių krypčių – augalinės kilmės junginių, pavyzdžiui, eterinių aliejų, taikymas, nes jiems būdingas atbaidomasis (Nerio ir kt., 2010), maitinimąsi slopinantis (Ebadollahi, 2011) ir insekticidinis poveikis (Ebadollahi ir Jalali Sendi, 2015). Eterinių aliejų veiksmingumas priklauso ir nuo vabzdžio rūšies, vystymosi stadijos ir nuo lyties, o dauguma tyrimų iki šiol apsiribojo kitais sandėliuojamų produktų kenkėjais, pavyzdžiui, mažuoju buožiaūsiu milčiumi (*Tribolium castaneum* (Herbst)) (Olivero-Verbel ir kt., 2013), nes jis laikomas modeliniu objektu ir tyrimų metodikos yra geriau standartizuotos. *Tenebrio molitor*, ypač lervų, elgsenos reakcijos tebėra menkai ištirtos ir nepakankamai aprašytos.

Be augalinės kilmės eterinių aliejų, natūralių atbaidančių medžiagų galima ieškoti ir sandėliavimo aplinkoje – grybais užkrėstuose grūduose. Kadangi grybais užkrėsti grūdai gali trikdyti *T. molitor* lervų vystymąsi ir didinti jų mirtingumą (Reiss, 1973), tikėtina, kad gebėjimas per atstumą atpažinti grybais užkrėstą substratą ir jo vengti pagal grybinės kilmės lakiuosius organinius junginius yra adaptyvus. Panašus grybų pažeistų substratų atpažinimas per atstumą nustatytas ištyrus pietinio ugniuko (*Plodia interpunctella* (Hübner)) elgseną. Tyrimo metu nustatyti šiam procesui

svarbūs bendri lakieji organiniai junginiai (Būda ir kt., 2016). *Tenebrio molitor* atveju vis dar trūksta tikslaus cheminių junginių identifikavimo ir veiksmingų dozių nustatymo, o tai riboja galimybes šiuos junginius praktiškai taikyti kenkėjų kontrolei ir elgsenos moduliavimui auginimo metu.

Dar viena *T. molitor* menkai ištirta cheminės ekologijos sritis – sėklų ir vabzdžių sąveikos. Sėklos gali išskirti įvairius cheminius signalus, kurie gali turėti poveikį kiaušinėlių dėjimui. Nors žinoma, kad augalų lakieji organiniai junginiai daro reikšmingą įtaką augalėdžių vabzdžių elgsenai ir augalų-vabzdžių sąveikoms tiek antžeminėje aplinkoje, tiek dirvožemyje (Johnson ir Rasmann, 2015), su sėklomis susiję chemoekologiniai mechanizmai *Tenebrio* genties vabalų atžvilgiu iki šiol neištirti. Ši spraga riboja supratimą apie vabzdžių kiaušinėlių dėjimo substratų pasirinkimą ir neleidžia kryptingai optimizuoti pramoninio jų veisimo. Sėklų junginių, kurie atbaido arba vilioja kiaušinėlius dedančias pateles, identifikavimas galėtų vienu metu pagerinti kenkėjų atbaidymo mechanizmus ir padidinti reprodukcijos efektyvumą auginimo sistemose.

Masinio auginimo ir sandėliavimo sąlygomis *T. molitor* ekologijai reikšmingą įtaką daro mikroorganizmai. Šiose aplinkose vabzdžiai susiduria su įvairiais grūdiniuose substratuose aptinkamais grybais ir mielėmis (Wakil ir kt., 2014; Cortes-Ortiz ir kt., 2016). Entomopatogeniniai grybai, pvz., *Metarhizium anisopliae* (Metchnikoff) ir *Beauveria bassiana* (Bals.-Criv.) Vuill., laikomi vienais pagrindinių infekcijų sukėlėjais pramoniniame vabzdžių auginime (Araújo ir Hughes, 2016; Hong ir kt., 2024), tačiau mielių įtaka šiuose kontekstuose yra menkai ištirta. Bendrai vabzdžių ir mielių sąveikos tebėra nepakankamai išnagrinėta vabzdžių ekologijos dalis.

Pastaruoju metu vis daugiau dėmesio skiriama žarnyno mikrobiotos įtakai vabzdžių elgsenai ir jų cheminiams signalams. Vabzdžių atmatos yra mikroorganizmams tinkama buveinė, galinti veikti šeimininko gyvybingumą, fiziologiją ir ekologiją (Magura ir kt., 2024). Žinoma, kad žarnyno mikrobiota prisideda prie maistinių medžiagų apykaitos, detoksikacijos, apsaugos nuo patogenų ir reprodukcijos (Engel ir Moran, 2013; Yang ir kt., 2021a,b; Wang ir kt., 2024a,b, 2025; Ding ir kt., 2024; Yasika ir Shivakumar, 2025). Kai kuriais atvejais ji taip pat gali būti semiocheminių signalų – feromonų ir kairomonų – šaltinis (Dillon ir kt., 2002; Sharon ir kt., 2011; Leroy ir kt., 2011a,b; Zhou ir kt., 2025). Nustatyta, kad kai kurioms vabzdžių rūšims atmatų lakieji organiniai junginiai veikia kaip agregaciją (susitelkimą) ir poravimosi partnerio priviliojimą skatinantys signalai (Davis ir kt., 2013), tačiau atmatų lakiųjų organinių junginių reikšmė *T. molitor* vabzdžių komunikacijai tebėra prastai suprantama. Lieka neaišku, ar suaugusių vabalų mikrobiotos sudėtis ir (ar) su ja susiję atmatų lakieji organiniai junginiai pasižymi lyčiai būdingais skirtumais. Trūksta tyrimų, kuriuose mikrobiotos

profilis būtų tiesiogiai susietas su elgsenos bandymais, siekiant įvertinti mikrobiotos poveikį ir galimus elgsenos skirtumus.

Tenebrio molitor elgesį gali formuoti įvairių cheminių signalų visuma: signalai iš augalinės kilmės medžiagų, naudojamų kaip maisto šaltinis ir kiaušinėlių dėjimo substratas, iš šias medžiagas kolonizuojančių grybų, taip pat iš paties vabzdžio organizmo – mikrobiotos. Šių įvairios kilmės signalų sąveika veikia vabzdžio elgesį aplinkoje, mitybą, kiaušinėlių dėjimą ir sąveikas su kitais organizmais, todėl iš esmės kuria rūšiai būdingą elgesio modelį. Šių signalų identifikavimas ir poveikio įvertinimas sudaro pagrindą kurti tikslingas priemones kenkėjų kontrolei bei augimo optimizavimui pramoninėse vabzdžių auginimo sistemose.

DARBO TIKSLAS IR UŽDAVINIAI

Darbo tikslas – nustatyti natūralios kilmės cheminius junginius, lemiančius kai kuriuos didžiojo milčiaus (*Tenebrio molitor* L.) lervų ir suaugėlių elgsenos ypatumus.

Uždaviniai tikslui pasiekti:

1. Įvertinti eterinių aliejų ir mikroskopiniais grybais užkrėstų grūdų – kaip natūralių lakiųjų organinių junginių mišinių – poveikį *Tenebrio molitor* elgsenai.
2. Iš atrinktų natūralių lakiųjų organinių junginių mišinių, pasižyminčių viliojančiu arba atbaidančiu poveikiu *T. molitor*, nustatyti pavienius junginius veikiančius elgseną.
3. Nustatyti, ar sandėliuojamų grūdų išskiriami lakieji organiniai junginiai veikia *T. molitor* patelių kiaušinėlių dėjimo elgseną, o aptikus poveikį – išskirti ir identifikuoti aktyviąsias medžiagas.
4. Įvertinti, ar *T. molitor* atmatų lakieji organiniai junginiai veikia kaip semiocheminės medžiagos, sukeliančios *T. molitor* vabalų tarpusavio elgsenos reakcijas.
5. Nustatyti laboratorinėje *T. molitor* kultūroje aptikto užkrėtimo sukėlėją(-us) ir įvertinti, ar užkrėtimas veikia išskiriamų lakiųjų organinių junginių sudėtį ir vabzdžių elgseną.

GINAMIEJI TEIGINIAI

1. *Tenebrio molitor* lervas repelentiškai veikia eterinių aliejų lakieji organiniai junginiai ir atskiri šių aliejų komponentai.
2. Suaugę *T. molitor* vabalai atpažįsta grybo *Aspergillus flavus* užkrėstus grūdus ir jų vengia.

3. Riebalų rūgštys (oleino, palmitino, linolo) reikšmingai slopina *T. molitor* patelių kiaušinėlių dėjimą.
4. Lakieji organiniai junginiai, iš *T. molitor* atmatų, veikia kaip semiocheminiai signalai vidurūšinėse sąveikose.
5. *Tenebrio molitor* žarnyno mikrobiotos sudėtis pasižymi lyčiai būdingais skirtumais.
6. *Tenebrio molitor* gali užsikrėsti mieliagrybiu *Hyphopichia burtonii*, kuris sumažina su reprodukcija susijusių feromonų gamybą ir pakeičia vabzdžio lakiųjų organinių junginių profilį, o padidėjęs limoneno kiekis yra tinkamas užkrėtimo žymuo.

DARBO NAUJUMAS IR AKTUALUMAS

1. Pirmą kartą identifikuotos natūralios kilmės medžiagos, veikiančios *Tenebrio molitor* elgseną: penki repelentiškai veikiantys eteriniai aliejai ir aštuoni pavieniai elgseną veikiantys lakieji organiniai junginiai, išskirti iš sudėtinių lakiųjų organinių medžiagų mišinių, kurie sudaro pagrindą elgsenos valdymui ir tvariai alternatyvai insekticidams.
2. Pirmą kartą nustatyta, kad *T. molitor* vabalai atpažįsta *Aspergillus flavus* užkrėstų grūdų lakiuosius organinius junginius ir jų vengia.
3. Pirmą kartą įrodyta, kad riebalų rūgštys veikia kiaušinėlių dėjimo substrato pasirinkimą – gauti nauji duomenys padeda optimizuoti substratus pramoniniam *T. molitor* auginimui.
4. Pirmą kartą nustatyta, kad *T. molitor* vabalų atmatų skleidžiami lakieji organiniai junginiai veikia kaip cheminiai signalai, tai atveria naujas populiacijos reguliavimo ir elgsenos kontrolės strategijas.
5. Pirmą kartą nustatyti reikšmingi suaugusių *T. molitor* vabalų žarnyno mikrobiotos skirtumai tarp lyčių.
6. Pirmą kartą aptiktas mieliagrybio *Hyphopichia burtonii* užkrėtimas *T. molitor* kolonijose.
7. Pirmą kartą įrodyta, kad *H. burtonii* užkrėtimas keičia *T. molitor* išskiriamų lakiųjų organinių junginių profilį, didindamas limoneno kiekius, tai kuria ankstyvos diagnostikos galimybę mikrobiologinės taršos monitoringui.

LITERATŪROS APŽVALGOS APIBENDRINIMAS

Literatūros apžvalgoje glaustai pateikta informacija apie didžiojo milčiaus (*Tenebrio molitor*) atliktų tyrimų istoriją ir svarbą, detalesnių tyrimų poreikį bei pagrindinius pasiekimus šioje srityje.

MEDŽIAGA IR METODAI

Tyrimų metu buvo taikomi įvairūs metodai. Detalus darbe taikytų metodų aprašymas yra pateiktas straipsniuose (**I-V straipsniai)**, kurių pagrindu ši disertacija yra parengta. Toliau pateikiami pagrindiniai naudotų tyrimo metodų aprašymai.

Vabzdžiai

Tyrimuose naudotos *Tenebrio molitor* lervos iš laboratorinės kultūros, palaikomos Valstybinio mokslinių tyrimų instituto Gamtos tyrimų centro (Vilnius) Cheminės ekologijos ir elgsenos laboratorijoje nuo 2019 m.

Lervos augintos skirtinguose substratuose, priklausomai nuo tyrimo: avižų dribsniai (70 %), kviečių sėlenos (20 %) ir sausos mielės (10 %) su morkomis (**I straipsnis**); kviečių sėlenos su morkomis (**II straipsnis**); kviečių sėlenos (90 %) ir sausos mielės (10 %) su morkomis (**III-V straipsniai**). Kai kuriuose tyrimuose maistinės mielės buvo naudojamos kaip maisto papildas, siekiant praturtinti *T. molitor* racioną. Visi tyrimai atlikti 25-26 °C temperatūroje, esant 60 ± 10 % santykinei oro drėgmei ir 12:12 h šviesos-tamsos režimui.

Elgsenos bandymams naudotos vidutinio ūgio lervos (14-20 mm; **I straipsnis**) ir lytiškai subrendę 7-15 dienų suaugę vabalai (**II-V straipsniai**); vabalų lytis nustatyta lėliukėms pagal paskutinio pilvelio segmento lytinius požymius (**II-V straipsniai**).

Žarnyno mikrobiotos analizei (**IV straipsnis**) atsitiktinai atrinkta 40 suaugusių 10-15 dienų amžiaus *T. molitor* vabalų. Vabzdžiai buvo eutanazuoti, ne trumpiau kaip 1 val. laikant juos –20 °C temperatūroje, o išėmus iš šaldiklio nedelsiant išskrosti. Prieš skrodimą vabalai 1 min. sterilizuoti 75 % etanolyje ir tris kartus nuplauti steriliu distiliuotu vandeniu. Kiekvieną mėginį sudarė penkių individų žarnynas su jo turiniu, patinų ir patelių mėginiai ruošti atskirai. Žarnų mėginiai surinkti į mėgintuvėlius ir laikyti iki tolesnės analizės.

Tyrimo medžiaga

I straipsnyje tirti šeši eteriniai aliejai (vieni pigiausių ir lengviausiai įsigyjamų) naudojant tris jų koncentracijas 0,05; 0,5; 5 % (tirpiklis heksanas): čiobrelių (*Thymus vulgaris*), eukaliptų (*Eucalyptus globulus*), šaltmėčių (*Mentha spicata*), levandinų (*Lavandula × hybrida*), citrinžolės (*Cymbopogon flexuosus*) ir gvazdikėlių (*Syzygium aromaticum*).

Naudoti analitiniai standartai (*R*)-(−)-karvonas, limonenas, mircenas, γ-terpinenas, *cis*-sabineno hidratas, terpinen-4-olis (**I straipsnis**), stearino, palmitino, linolo, oleino riebalų rūgštys (**II straipsnis**), 1-okten-3-olis,

heksanalis, 3-metilbutanolis, 1-pentanolis, toluenas, 1-heksanolis, 2-metilfuranas (**V straipsnis**). Pavienių junginių tyrimo metu naudotas heksanas kaip tirpiklis, o junginiai tirti trimis koncentracijomis: 0,05; 0,5 ir 5 %.

II straipsnyje kiaušinėlių dėjimo tyrimui naudoti avižų dribsniai (sumalti ir persijoti, 1 mm), kvietiniai ir rapsų miltai bei kviečių sėlenos, kvarcinis smėlis (0,0-0,4 mm) prieš bandymus išplautas ir 2 h kaitintas 150 °C. Taip pat tirta riebalų rūgščių įtaka 0,05%, 0,5% ir 5% koncentracijų rėžyje. **IV straipsnyje** mieliagrybio *Hyphopichia burtonii* izoliatai auginti standartinėse terpėse (bulvių dekstrozės ir salyklo agaras), o vabzdžių užsikrėtimo tyrimui paruošti substratai: sterilus agaras (kontrolė) ir agaras su sporuliuojančia *H. burtonii* arba entomopatogeniniu grybu *Metarhizium anisopliae*.

V straipsnyje *Aspergillus flavus* kultūra auginta bulvių dekstrozės agaro terpėje 25 °C temperatūroje 7 dienas (iki sporuliacijos), o terpės ir grūdų mėginiai prieš užkrėtimą sterilizuoti autoklavu (125 °C, 1,2 atm). Visi darbai atlikti sterilioje II klasės spintoje. Sterilūs 2 g grūdų mėginiai užkrėsti sporomis nuo 7 dienų amžiaus kolonijų, sudrėkinti 300 µl steriliu vandeniu ir auginti 0, 5, 10 bei 20 dienų 27 °C temperatūroje.

Elgsenos tyrimai

Dviejų pasirinkimo testas atliktas 10,5 cm Petri lėkštelėse su filtriniu popieriumi: viena periferinė pusė apdorota eteriniu aliejumi arba pavieniu junginiu, kita – tirpikliu (kontrolė). Naudota 10 µL (5×2 µL) stimulo, stebėtas elgesys 5 min, judėjimas analizuotas „EthoVision XT 12“ vaizdo registravimo ir analizės programa (**I ir V straipsniai**).

Dviejų pasirinkimų duobelinės gaudyklės (angl. *pitfall*) tipo testas naudotas *H. burtonii*, *Aspergillus flavus* bei atmatų lakiųjų organinių junginių cheminiams signalams įvertinti (**III-V straipsniai**).

Kiaušinėlių dėjimo tyrime (**II straipsnis**) naudotos tinkleliu dengtos dėžutės su 20 g substrato, įleista po 10 patinų ir 10 patelių (su antenomis arba be jų), vandens šaltinis – morkos, po 24 h suskaičiuoti padėti kiaušinėliai.

Mirtingumo tyrime lygintas *H. burtonii* ir *M. anisopliae* patogeniškumas lervoms (po 25 lervas grupėje): 200 µL sporų suspensijos (1×10^7 sporų/mL) užlašinta ant 0,5 cm agaro gabalėlio; lervos 3 dienas maitintos užkrėstu agaru, vėliau pridėta 0,5 g sterilių kviečių sėlenų; išgyvenamumas stebėtas 30 dienų (**IV straipsnis**).

Cheminė analizė

Lakiųjų organinių junginių identifikavimas atliktas GC-MS metodu (Shimadzu GC/MS-Q2010 PLUS, Rxi-5 Sil MS kolonėlė), junginius

identifikuojant pagal sulaikymo indeksus ir masių spektrų bibliotekas (≥ 90 % atitikmuo) (**I, IV ir V straipsniai**).

Molekuliniai tyrimai

Žarnyno mėginiai buvo pateikti „ZymoBIOMICS Targeted Sequencing Service“ (Zymo Research, Irvine, CA) 16S rRNR geno V3-V4 regiono sekoskaitai ir bioinformatinei analizei, įskaitant mikrobinės DNR išskyrimą su kontroliniais mėginiais, sekoskaitą ir duomenų apdorojimą (taksonominė priskirtis naudojant „Zymo Research“ 16S duomenų bazę, alfa ir beta įvairovės analizė naudojant Qiime v.1.9.1; LEfSe taikant linijinę diskriminantinę analizę (LDA)) naudojant „Zymo Research“ 16S duomenų bazę (**III straipsnis**). Mieliagrybio genominė DNR buvo išskirta modifikuotu CTAB metodu, sekos identifikuotos ir sulygintos su tipinėmis padermėmis ir filogeniškai analizuotos maksimaliojo tikėtinumo metodu, o gautos sekos pateiktos „GenBank“ (**IV straipsnis**).

Statistinė analizė

Statistinėje analizėje taikyti Vilkoksono porinių rangų (**I ir V straipsniai**) ir tikslus binominis testas (**III ir V straipsniai**), ANOVA su Tukey HSD paklaida, Friedmano ir Mann-Whitney U testai (**II straipsnis**), ANOVA/PERMANOVA/PCoA (**III straipsnis**) bei Kaplan-Meier ir log-rank (Mantel-Cox) analizė (**IV straipsnis**), reikšmingumo lygis $p < 0,05$.

REZULTATAI IR JŲ APTARIMAS

Visi rezultatai buvo aprašyti ir aptarti straipsniuose, kuriais paremta ši disertacija (**I-V straipsniai**). Toliau apibendrinami pagrindiniai disertacijos uždavinius atitinkantys rezultatai.

Tenebrio molitor reakcija į lakiuosius organinius junginius

Tiriant šešių (citrinžolių, čiobrelių, levandinų, gvazdikėlių, eukalipto ir pipirmėčių) eterinių aliejų poveikį *T. molitor* lervoms nustatyta, kad jos dažniausiai rinkosi kontrolinę zoną ir vengė eteriniu aliejumi apdorotos zonos (išimtis – eukalipto eterinis aliejus) ($p < 0,05$) (**I straipsnis**). Atbaidomasis poveikis skyrėsi tarp pačių aliejų: silpniausiai atbaidė citrinžolės, čiobrelių ir levandinų aliejai, vidutiniškai – gvazdikėlių, o stipriausiai – šaltmėčių aliejus. Bendras judėjimo slopinimas (pagal praleistą laiką stimulo zonoje) svyravo nuo 1,84 iki 5,74 karto, o įveiktas atstumas eterinio aliejaus (stimulo) zonoje sumažėjo apie 2,23-17,24 karto (**I straipsnis**). Dažniausiai stipriausias

poveikis vabzdžiams būdingas aliejams, kuriuose vyrauja karvonas ar eugenolis (pvz., šaltmėtės ir gvazdikėlių aliejuose), kaip nustatyta ankstesnių tyrimų duomenimis su kitais sandėliuojamų produktų kenkėjais (George ir kt., 2009; Martínez ir kt., 2018).

Cheminė analizė atskleidė, kad eterinių aliejų sudėtis labai heterogeniška (nevienoda): tik trys lakieji organiniai junginiai (limonenas, linalolis, *trans*-kariofilenas) buvo bendri visiems šešiems tirtiems eteriniams aliejams ir jų gausumas stipriai skyrėsi tarpusavyje (**I straipsnis**).

Pavienių junginių analizė parodė, kad stipriausią atbaidantį poveikį *T. molitor* lervoms sukėlė terpinen-4-olis (visos tirtos dozės 0,05-5 % reikšmingai sumažino įveiktą atstumą stimulo zonoje), o karvono sukeltas judėjimo slopinimas buvo priklausomas nuo dozės (veiklus tik 0,5-5 % koncentracijų rėžyje). *cis*-Sabineno hidratas statistiškai reikšmingai veikė lervų judėjimą tik didžiausios koncentracijos (5 %), kai tuo tarpu limonenas, mircenas ir γ-terpinenas reikšmingo poveikio lervų judėjimui iš vis neturėjo (**I straipsnis**).

Šie rezultatai rodo, kad eterinių aliejų mišiniuose elgesio atsaką gali keisti komponentų tarpusavio sąveikos (pvz., antagonizmas), dėl kurių bendras repelentiškumas gali būti silpnesnis, net jei mišinyje yra aktyvaus junginio. Tikėtina, kad toks poveikis stebimas su čiobrelio eteriniu aliejumi – atbaidantis poveikis buvo silpnesnis, nors jame aptikta stipriausiai lervų elgesį veikiančio terpinen-4-olio (Satyal ir kt., 2016; **I straipsnis**).

Be augalinės kilmės lakiųjų junginių, *T. molitor* elgseną veikė ir jų atmatos. Lakieji organiniai junginiai iš suaugusių *T. molitor* vabalų atmatų sukėlė aiškias elgesio reakcijas tarp lyčių (**III straipsnis**). *Tenebrio molitor* patinus reikšmingai viliojo tiek patinų tiek patelių atmatos, o *T. molitor* pateles viliojo tik patinų atmatos (**III straipsnis**). Šie duomenys rodo, kad atmatų lakieji organiniai junginiai yra ekologiškai svarbūs, kurie gali veikti vidurūšinę komunikaciją.

Ankstesnių tyrimų duomenys atskleidžia, kad *T. molitor* lervos telkiasi aplink savo atmatas, reaguodamos į kontaktinius signalus (Weaver ir kt., 1989). Šiame darbe su suaugusiais vabalais atlikti tyrimai, kuriuose vertinti lakieji junginiai, papildo iki šiol žinomus rezultatus ir leidžia daryti išvadą, kad skirtinguose vystymosi tarpsniuose gali būti naudojami skirtingi jutiminiai signalai.

Vienas iš tokių junginių šaltinių yra *Aspergillus flavus* užkrėsti grūdai. Lakieji organiniai junginiai iš *A. flavus* užkrėstų grūdų veikia atbaidančiai suaugusius *T. molitor* vabalus, o tai reiškia, kad jie geba atskirti užkrėstus šiuo grybu grūdus nuo neužkrėstų. Patelės užuodžia užkrėstus grūdus nuo pirmos užkrėtimo dienos, o patinai nuo penktos (**V straipsnis**). Yra žinoma, kad grybais užkrėsti grūdai neigiamai veikia *T. molitor* lervų vystymąsi ir gali būti joms toksiški (Reiss, 1973).

Pavienių junginių duomenų analizė atskleidė nuo lyties priklausomas reakcijas: 1-okten-3-olis buvo stipriai atbaidantis patinus nuo 0,05 % koncentracijos, bet patelių šis junginys statistiškai patikimai neveikė (**V straipsnis**). Heksanalis atbaidė patinus tik nuo ≥ 0,5 %, o patelės šiam junginiui buvo jautresnės – veiklios visos tirtos koncentracijos (0,05-5 %). Visos tirtos 1-heksanolio dozės pateles viliojo, o patinams šis junginys buvo repelentiškas tik didžiausioje dozėje (5 %). Heksanalis, yra žinomas kaip gausus junginys nesubrendusiuose grūduose ir bręstant jo kiekis mažėja – tikėtina šis elementas *T. molitor* veikia kaip šviežių grūdų (netinkamų maistui) indikatorius (Ji ir kt., 2017; Būda ir kt., 2016). Be to, priešingos 1-heksanolio savybės (patelių viliojimas ir patinų atbaidymas) rodo, kad galutinis vabzdžio elgesys priklauso būtent nuo mišinio sudėties (**V straipsnis**).

Junginiai, veikiantys *Tenebrio molitor* patelių kiaušinėlių dėjimą

Tenebrio molitor patelių kiaušinėlių dėjimą lemia ne vien substrato maistinė vertė, bet ir jame esantys cheminiai junginiai, keisdami dėjimo vietos pasirinkimą ir padėtų kiaušinėlių kiekį.

Patelės (su antenomis ir be jų) dėjo reikšmingai daugiau kiaušinėlių į avižų ir kviečių miltus nei į rapsų miltus ar kviečių sėlenas ($p < 0,05$) (**II straipsnis**). Antenų pašalinimas kiaušinėlių dėjimo vietos pasirinkimo nepakeitė, bet padidino variaciją. Mažiausias dėjimas išliko į kviečių sėlenas (**II straipsnis**). Kad būtų atskirtas cheminis poveikis nuo fizinių savybių, kvarcinis smėlis (SiO_2) pasirinktas kaip neutralus substratas: jame patelės dėjo apie 78 % kiaušinėlių, lyginant su kviečių ir avižų miltais (**II straipsnis**).

Smėlį sumaišius su avižų ekstraktu, patelės padėjo tik apie 4 % kiaušinėlių, palyginti su grynu smėliu. Šį efektą greičiausiai lemia tai, kad neapdorotuose avižų miltuose pateles veikia visa teigiamų ir neigiamų cheminių bei fizinių signalų visuma, o smėlyje su avižų ekstraktu išlieka tik ekstrakte esantys inhibitoriniai junginiai, todėl kiaušinėlių dėjimas yra slopinamas. Avižų ekstraktą frakcionavus į dvi frakcijas nustatyta, kad abi frakcijos, sumaišytos su ekstrahuotomis avižomis (be dalies lakiųjų organinių junginių), sumažino kiaušinėlių dėjimą, lyginant vien su ekstrahuotomis avižomis ($p < 0,05$). Šis poveikis buvo statistiškai reikšmingas tik patelėms su nepažeistomis antenomis, todėl tai rodo, kad kiaušinėlių dėjimą slopinantys signalai bent iš dalies suvokiami antenų jutiminių organų pagalba (**II straipsnis**). Stiprus avižų ekstrakto ir jo frakcijų slopinimas rodo, kad kiaušinėlių dėjimą gali reguliuoti keletas semiocheminių medžiagų, įskaitant lakiąsias organines medžiagas (Tasin ir kt., 2011; Lei ir kt., 2024).

Pastebėjus, kad avižų ekstrakte išryškėja didelis riebalų kiekis, ištirtos keturios gausios riebalų rūgštys, esančios grūduose (sėklose): linolo, oleino, palmitino ir stearino (**II straipsnis**). Nustatyta, kad riebalų rūgštys (0,5 % ir 5

% koncentracijos) statistiškai reikšmingai sumažino padėtų kiaušinėlių skaičių ($p < 0,05$), t. y. poveikis pasireiškė ne vien kiaušinėlių pasiskirstymu tarp pasirinkimų, bet ir padėtų kiaušinėlių skaičiaus sumažėjimu. Tai rodo ne tik dėjimo vietos vengimą, bet ir kiaušinėlių dėjimą slopinantį poveikį (**II straipsnis**).

Dėjimo vietos vengimas buvo priklausomas ir nuo junginio bei dozės: reikšmingai kiaušinėlių dėjimą slopino 5 % oleino ir linolo bei 0,5 % linolo ir palmitino rūgštys, o visų tirtų rūgščių mažiausia (0,05 %) dozė neturėjo slopinamojo poveikio *T. molitor* patelių kiaušinėlių dėjimui (**II straipsnis**). Žinant substratų riebalų rūgščių sudėtį (pvz., didesnė oleino ar linolo dalis rapsuose, reikšminga linolo dalis kviečiuose ar avižose), galima tiksliau prognozuoti kiaušinėlių dėjimo intensyvumą ir optimizuoti veisimo sąlygas (Kouřimská ir kt., 2018; Lidon ir kt., 2019; Chew, 2020).

Tenebrio molitor mikroorganizmai

Mikroorganizmai gali veikti *T. molitor* vabalų elgseną netiesiogiai, nes žarnyno mikrobiota yra priklausoma nuo lyties ir/arba rūšies ir gali keisti vabzdžio išskiriamų kvapų cheminę sudėtį per metabolinius junginius, be to, su vabzdžiu susiję mikroorganizmai, įskaitant mikrobiotą, gali keisti jo lakiųjų organinių junginių emisijas (**III ir IV straipsniai**).

Tenebrio molitor žarnyno mikrobiotos alfa ir beta įvairovės analizė atskleidė reikšmingus vidurūšinius lyčių skirtumus. *Tenebrio molitor* patelių žarnyne mikroorganizmų bendrijos rūšių gausumas ir tolygumas (Shannon ir Simpson indeksai) buvo reikšmingai ($p = 0,009$ ir $p = 0,02$ atitinkamai) didesnis nei patinų (**III straipsnis**). Taip pat buvo įvertintas *T. molitor* patinų bei patelių žarnyno mikrobiotos sudėties santykinis gausumas įvairiuose taksonominiuose ranguose. *Spiroplasma* genties bakterijos (tipas Mycoplasmatota – klasė Mollicutes – eilė Entomoplasmatales – šeima Spiroplasmataceae) buvo identifikuotos tik *T. molitor* patinų žarnyno mėginiuose, kur jos sudarė 43,2 %, tuo tarpu *Enterococcus* genties bakterijos (tipas Bacillota – klasė Bacilli – eilė Lactobacillales – šeima Enterococcaceae) vyravo patelių žarnyno mikrobiotoje ir sudarė 51,7 % (**III straipsnis**). Vabalų žarnyno mikrobiotos lytinis dimorfizmas atitinka kitų vabalų tyrimuose aprašytus dėsningumus ir gali veikti cheminę komunikaciją, nes mikroorganizmų gaminami metabolitai, lakūs organiniai junginiai gali keisti šeimininko kvapų profilį (Engel ir Moran, 2013; Engl ir Kaltenpoth, 2018; Wan ir kt., 2020).

Tokio poveikio galimybę patvirtina ir šiame darbe nustatyti su mikroorganizmais susiję pokyčiai laboratorinėse *T. molitor* populiacijose. Auginant *T. molitor* vabzdžius laboratorijoje buvo nustatytas su mikroorganizmais siejamas užkrėtimas, paveikęs tiek lervas, tiek suaugusius

vabalus. Užkrėtimas pasireiškė matoma išorine kolonizacija bei sumažėjusiu vabzdžių judrumu (**IV straipsnis**). Stebimos baltos apnašos ties burnos dalimis ir kūno nareliais, vėliau – vangumas, silpnesnis reagavimas, kai kuriems vabalams ribotas judėjimas.

Molekuliniai duomenys patvirtino, kad užkrėtimas buvo sukeltas mieliagrybio *Hyphopichia burtonii* (**IV straipsnis**). Tai svarbu, nes mielės yra menkai aprašytos tarp gerai žinomų masiškai auginamų vabzdžių ligų sukėlėjų, nors auginimo aplinka ir grūdiniai substratai sudaro sąlygas oportunistiniam mikroorganizmų plitimui (Davis, 2015; Bernardi ir kt., 2019; Slowik ir kt., 2023). Mieliagrybis *H. burtonii* nebuvo letalus *T. molitor* lervoms: per 7 dienų stebėjimo laikotarpį mirtingumo nenustatyta nei kontrolinėje, nei mielėmis užkrėstų lervų grupėje, tačiau, kai lervos buvo veikiamos entomopatogeninio grybo *M. anisopliae*, iki 7 dienos žuvo visos lervos (**IV straipsnis**). Tai rodo, kad mieliagrybio poveikis buvo subletalus – jis galėjo silpninti lervas ar bloginti jų būklę, bet per šį laikotarpį nesukėlė mirtingumo.

Nustatyta reikšmingai pakitusi lakiųjų organinių junginių sudėtis vabzdžiams, kurie buvo užkrėsti *H. burtonii*, palyginti su sveikais tos pačios rūšies individais (**IV straipsnis**). Stebėtas vabzdžio feromonų išskyrimo mažėjimas užkrėstuose individuose. Užkrėstų *T. molitor* lervų lakiųjų organinių junginių profilyje nustatytas ryškiai padidėjęs limoneno kiekis, o sveikų lervų emisijose šis junginys nebuvo aptiktas. Tai rodo, kad limonenas gali būti laikomas potencialiu užkrėtimo žymeniu (**IV straipsnis**).

Bendrai, užkrėstų lervų lakiųjų organinių junginių mišinys pasižymėjo didesniu junginių skaičiumi (identifikuoti 23 junginiai), palyginti su sveikomis lervomis (identifikuoti 16 junginių). Suaugusių vabalų organinių junginių profilis buvo mažiau turtingas nei lervų, tačiau taip pat junginių mišinys pasižymėjo didesniu junginių skaičiumi užkrėstuose individuose (identifikuoti 13 junginių), lyginant su sveikais (identifikuoti 10 junginių) (**IV straipsnis**). Tiriant suaugusių vabalų reakciją į sveikas ir *H. burtonii* užkrėstas lervas, jokių vengimo požymių nebuvo nustatyta ($p > 0,05$). Tai rodo, kad pakitę užkrėstų lervų kvapų signalai nesukelia jokios suaugėlių reakcijos, todėl kontaktas tarp individų išlieka ir užkrėtimas gali sėkmingai plisti kolonijoje (**IV straipsnis**).

IŠVADOS

1. Šie lakūs organinių junginių (LOJ) mišiniai pasižymi repelentiniu poveikiu *Tenebrio molitor*:
    a. lervoms – stipriausiu repelentiniu poveikiu pasižymi šaltmėčių (*Mentha spicata*) eterinių aliejų LOJ, vidutiniu – gvazdikėlių (*Syzygium aromaticum*), o silpniausiu – citrinžolės (*Cymbopogon*

flexuosus), čiobrelių (*Thymus vulgaris*) ir levandinų (*Lavandula* × *hybrida*) eterinių aliejų LOJ;

b. suaugusiems vabalams – grybu *Aspergillus flavus* užkrėstų grūdų LOJ.

2. LOJ mišiniuose identifikuoti aštuoni pavieniai junginiai, darę poveikį *T. molitor* suaugėlių ir lervų elgsenai:
    a. šeši repelentinėmis savybėmis pasižymintys junginiai (karvonas, *cis*-sabineno hidratas, terpinen-4-olis, 1-okten-3-olis, heksanalis, 1-heksanolis);
    b. vienas atraktantas (3-metilbutan-1-olis);
    c. vienas junginys, kurio poveikis priklausė nuo lyties: pateles viliojo, patinus atbaidė (1-heksanolis).
3. *Tenebrio molitor* patelių kiaušinėlių dėjimą slopina substrato riebalų rūgštys: palmitino ir linoleno, kai koncentracija ≥ 0,5%, ir oleino, kai koncentracija ≥ 5%.
4. Lakieji organiniai junginiai iš *T. molitor* atmatų veikia vidurūšines sąveikas: patinų atmatos veikia agregaciją (vilioja savo rūšies pateles ir patinus), o patelių atmatos vilioja patinus. Žarnyno mikrobiotos sudėtis taip pat skiriasi priklausomai nuo vabalų lyties: *T. molitor* patinų mikrobiotoje gausiausia buvo *Spiroplasma*, patelių – *Enterococcus*.
5. Plačiai paplitęs mieliagrybis *Hyphopichia burtonii* gali užkrėsti *T. molitor* kultūrą, dėl ko mažėja vabzdžio feromonų išskyrimas ir pakinta lakiųjų organinių junginių sudėtis: ypač reikšmingai padidėja limoneno, todėl jis yra tinkamas žymuo ankstyvam užkrėtimui aptikti.

ADDITIONAL PAPERS PUBLISHED DURING THE PHD STUDIES (NOT INCLUDED IN THIS THESIS)

Publications in journals with an impact factor and referred in the Clarivate Analytics Web of Science database.

- Būda, V., Radžiutė, S., Apšegaitė, V., Blažytė-Čereškienė, L., Čepulytė, R., **Bumbulytė, G**. and Mozūraitis, R., 2022. Electroantennographic and behavioral responses of European cherry fruit fly, *Rhagoletis cerasi*, to the volatile organic compounds from sour cherry, *Prunus cerasus*, fruit. *Insects*, *13*(2), p.114. DOI: 10.3390/insects13020114 (IF 3, Q1 quartile);
- Radžiutė, S., Orlovskytė, S., Būdienė, J., Blažytė-Čereškienė, L., **Bumbulytė, G**. and Būda, V., 2023. Stored product pest *Nemapogon variatella* (Clemens): Identification of sex pheromone, putative antagonist and mitotype characterization. *Journal of Applied Entomology*, *147*(9), pp.857-867. DOI: 10.1111/jen.13178 (IF 1,7, Q2 quartile).

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CURRICULUM VITAE

gabriele.bumbulyte@gamtc.lt

EDUCATION

2015 – 2019 **Vilnius University**
Bachelor of Biology

2019 – 2021 **Vilnius University**
Master of Biodiversity

PUBLICATIONS

Scientific

- Būda, V.; Radžiutė, S.; Apšegaitė, V.; Blažytė-Čereškienė, L.; Čepulytė, R.; **Bumbulytė, G.**; Mozuraitis, R. Electroantennographic and Behavioral Responses of European Cherry Fruit Fly, *Rhagoletis cerasi*, to the Volatile Organic Compounds from Sour Cherry, *Prunus cerasus*, Fruit. Insects 2022, 13, 114. https://doi.org/10.3390/insects13020114
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- **Bumbulytė-Žukevičienė**, G., Lučinskaitė, I., Čepukoit, D., Būda, V. (2024-11-26). Wheat grain infected by *Aspergillus flavus* modifies behavior of yellow mealworm, *Tenebrio molitor* L. The 13th International Conference YOUNG SCIENTISTS FOR ADVANCE OF AGRICULTURE AGRISCI2024: abstract book p. 56, Vilnius, Lithuania
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  http://www.njmk.smm.lt/lt/naujienos/32/;
- **Bumbulytė, G.,** Petreikis, L., (2015-09-17). Herbal Extracts to Control Granary Weevil (*Sitophilus granarius* L.). Europos Sąjungos jaunųjų mokslininkų konkurse atstovauta Lietuva ir laimėtas specialusis prizas – tyrimo tobulinimo kelionė į Joint Research Centre Isproje (Italija); https://www.smm.lt/web/lt/pranesimai_spaudai/naujienos_1/es-jaunuju-moksliininku-konkursepuikus-musu-atstovu-pasiekimai;
- Winner of the prize in the 2019-02-19 University Students' Research Paper Competition for the paper "Effect of essential oils on the larvae

of the pest Tenebrio molitor L. (Tenebrio molitor L.), a major pest of agricultural products in storage". http://www.lma.lt/archyvas-2019-m;

- 2021-11-18. Awarded as the author of the best Master's thesis in the field of agricultural sciences in the Best Master's Thesis Competition organised by the Lithuanian Union of Young Scientists (LJMS) and the Ministry of Foreign Affairs (MoFA). Her thesis "Search for compounds repelling the melaworm (*Tenebrio molitor* L.) in essential oils of *Thymus vulgaris* L., *Mentha spicata* L. and metabolites of microscopic fungi" https://naujienos.vu.lt/vilniaus-universiteto-absolventai-apdovanoti-uz-geriausius-magistro-darbus/?fbclid=IwAR3KSRTWmwVvgoN5ESjh-IyKIFoF2ATx_dz7f8i7L8RDSDHff2MF-AaXiAQ
- In the International Conference of Life Sciences, the COINS **Bumbulytė G**. was awarded the most entrepreneurial poster by the LRC. https://www.linkedin.com/posts/gabriele-bumbulyte-2989241b3_coins2023-lithuanianresearchcouncil-activity-7062441251270193153-2XpV?utm_source=share&utm_medium=member_desktop
- **Bumbulytė**, G. (2024-07). ISCE Student Travel Award. Prague, Czechia
- **Bumbulytė**, G. (2024-11-26). Diploma for the best presentation in the 13th International Conference YOUNG SCIENTISTS FOR ADVANCE OF AGRICULTURE AGRISCI2024
- Research council of Lithuania. Scholarship for academic achievements: 2024 (award no. P-DAP-24-98).
- Research council of Lithuania. Support to attend the the 37th annual International Society of Chemical Ecology meeting in Malaysia (2022, award no. P-DAK-22-89).
- Research council of Lithuania. Support to attend the the 39th annual International Society of Chemical Ecology meeting in Czech Republic (2024, award no. P-DAK-24-51).
- **Bumbulytė-Žukevičienė**, G. (2025-04-16/17). Innovative Research Award at the International Food for Life Conference.
- **Bumbulytė-Žukevičienė**, G. (2025-10-10). Nacionalinės atrankos InSPiR2eS Global Pitching Research Competition (IGPRC2025) konkurso nugalėtoja.

POJECTS

- The Research Council of Lithuania, Student Summer Internships (student Arijus Auškalnis), 2025, award no. P-SV3-25-459

RESEARCH TRAINEESHIP

- In 2019, the LMT-funded competitive student research traineeship "Possible influence of hygienic behavior of bees on the prevalence of viruses in honeybee colonies" was carried out. Project No. 09.3.3.-LMT-K-712-15-0104.
- "Search for repellent chemical compounds in the essential oils of mint (*Mentha spicata*), thyme (*Thymus vulgaris*) for mealworm (*Tenebrio molitor*)" - a competitive student research traineeship funded by the LMT in 2019. Project No 09.3.3.-LMT-K-712-16-0204.
- "Search for repellent chemical compounds in volatile metabolites of microscopic fungi for the mealworm (*Tenebrio molitor* L.)", a competitive student research traineeship funded by the LMT and carried out in 2020. Project No 09.3.3.-LMT-K-712-22-0225.
- Training for identification of chemical pheromones at the Institute of Organic Chemistry, Technische Universitat Braunschweig (Germany), 2024-05-27 - 2024-05-31.

BACHELOR STUDENTS' SUPERVISION

2023-2024 Vilnius University, biology program

- Ieva Lučinskaitė
- Arijus Auškalnis
- Justinas Sadauskas

2024-2025 Higher education institution, Agribusiness technologies program

- Aušrinė Pupšytė

2024-2025 Vilnius University, biology program

- Fausta Molotokaitė

MASTER STUDENTS' SUPERVISION

2024-2025 Vilnius University, biodiversity program

- Felicija Kvedaraitė

2024-2025 Vilnius University, microbiology program

- Arijus Auškalnis

SCIENTIFIC COMPETENCE

Working with the Ethovision XT video-tracking system for recording animal behavior; other general techniques for insect behavioral studies; techniques for various insect field trials and preparations; working with electroantenogram (EAG) recording; gas chromatography-electroantenographic detection (DC-EAG); insect growing and care.

EXTERNAL PROFESSIONAL ACTIVITIES

2021–Present – Member, International Society of Chemical Ecology (ISCE)
2025–Present – Member, Ecological Society of America (ESA)
2026–Present – Member, Entomological Society of America (ESA)

CERTIFICATES

- Introduction to R (1.25 ECTS). The training took place on 15 and 18 December 2023.
- Electronic scientific information resources (0.2 ECTS). The training took place on 16 November 2023.
- Developing open access competencies (0.5 ECTS). The training took place on 10 November 2023.
- Introduction to research data management (0.15 ECTS). The training took place on 8 November 2023.
- Information management and citation using "Zotero" (0.15 ECTS). The training took place on 5 April and 22 April 2024.
- Introductory data analysis in R: a language and environment for statistical computing (20 hours). The training took place on November 11th – 15th 2024.

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PAPER I

Essential oils and their components control behavior of yellow mealworm (*Tenebrio molitor*) larvae

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Article

Essential Oils and Their Components Control Behaviour of Yellow Mealworm (*Tenebrio molitor*) Larvae

Gabrielė Bumbulytė, Jurga Būdienė and Vincas Būda *

Laboratory of Chemical and Behavioural Ecology, Nature Research Centre, Akademijos Str. 2, LT-08412 Vilnius, Lithuania; gabriele.bumbulyte@gamtc.lt (G.B.); jurga.budiene@gamtc.lt (J.B.)
* Correspondence: vincas.buda@gamtc.lt

Simple Summary: This study investigated the use of essential oils (EOs) from different plants to control the behaviour of *Tenebrio molitor* L., a pest of grain and flour. Among the EOs tested, spearmint was the most effective repellent for mealworm larvae, followed by clove. East-Indian lemongrass, thymus, lavandin, and eucalyptus EOs showed lower repellent properties. Terpinene-4-ol and carvone, were the most effective repellents, while limonene, myrcene, and γ-terpinene had no significant activity.

Abstract: Beetle *Tenebrio molitor* L. (Coleoptera, Tenebrionidae) is a well-known pest of grain and flour in food stores and grocery shops. Recently, commercial cultivation of the insect was started for human food and animal feed. Behaviour control of this insect using natural repellents is promising both for grain protection and commercial cultivation. We analysed if natural products of plant origin, namely essential oils (EOs), could be used for this purpose. Behavioural tests were performed using EOs of six plants: thymus (*Thymus vulgaris*), eucalyptus (*Eucalyptus globulus*), spearmint (*Mentha spicata*), lavandin (*Lavandula* × *hybrida*), East-Indian lemongrass (*Cymbopogon flexuosus*), and clove (*Eugenia caryophyllus*). The most effective repellent for mealworm larvae was EO of spearmint, moderate activity showed that of clove and the least repellent were EOs of lemongrass thymus and lavandin. EO of eucalyptus caused almost no or very low effect. Six of the most abundant compounds of the EOs were selected for testing. The most effective single compounds were terpinene-4-ol and carvone, low-effective cis-sabinene hydrates and those of no significant activity were limonene, myrcene and γ-terpinene.

Keywords: insect behaviour; repellent; stored-food insect; bioassay; pest control

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1. Introduction

Grain products are among the main sources of human food and feed for domestic animals. During grain storage, 5–20% of the harvest is lost worldwide due to insects [1]. This is related to several insect species whose main source of feed is stored dry grains.

In warehouses, grain pests can reproduce throughout the year, causing damage and losses in both grains and their products, as well as deteriorating their quality. These pests can breed not only in storage facilities with stored products but also in empty warehouses, silos, grain transportation vehicles, and grain residues in cracks in the floors and walls. Pest-contaminated feed that enters farms is a source of further pest spread.

Chemical insecticides are used for grain protection [2]. However, using insecticides widely has caused many negative effects both on the environment and on human health. Therefore, search for alternative pest control methods is required, and the application of natural compounds, especially plant-based ones, could be of great importance.

The yellow mealworm (*Tenebrio molitor* L.) is among those economically important insect pests that damage grain and flour a lot. Therefore, this species was chosen for the search for environmentally friendly means suitable for behavioural control. It has been revealed that insects of this species are distinguished by relatively high biomass growth, its larvae are

nutritious and a rich source of protein, and it can be easily and extensively cultivated for human food and animal feed. With the development and/or improvement of technologies for the cultivation of this insect species, data on behavioural control are relevant.

Understanding the biology, behaviour, and ecology of *T. molitor* as a pest is crucial for developing effective control measures to ensure grain protection and food safety. The aim of this paper was to search for essential oils (EOs) (harmless to the environment, safe for humans) and their components that affect the behaviour of *T. molitor* larvae.

2. Materials and Methods

2.1. Insects

T. molitor larvae used in the tests were obtained from a culture maintained at the Laboratory of Chemical Ecology and Behaviour, Nature Research Centre, since 2019. The larvae were reared on a diet consisting of a mixture of oat flakes (70%), wheat bran (20%), and dry yeast (10%), and provided with carrot slices. During rearing, a temperature of 25 °C, a relative humidity of 60%, and a 12:12 h light–dark cycle was maintained. For the experiments, medium-sized larvae (14–20 mm in length) were selected.

2.2. Essential Oils and Chemicals of and Identification of Individual Components

The essential oils (EOs) used in the tests were purchased from "JSC Naujoji Barmune", a retailer of EOs in Lithuania. The following EOs were used: thyme (*Thymus vulgaris*), eucalyptus (*Eucalyptus globulus*), spearmint (*Mentha spicata*), lavandin (*Lavandula* × *hybrida*), East-Indian lemongrass (*Cymbopogon flexuosus*), and clove (*Eugenia caryophyllus*). All EOs were labelled with the common and scientific names of the plant species they were extracted from, as well as with series numbers and expiry dates. However, the origin of the plants used to extract the EOs was not indicated.

Analytical standards used in the experiment were purchased from different companies: (R)-(−)-carvone from Toronto Research Chemicals Inc. (Toronto, ON, Canada); limonene (sum of enantiomers, analytical standard, ≥98% (GC)), myrcene (analytical standard, ≥95% (GC)), and γ-terpinene (analytical standard, ≥97% (GC)) from Fluka (Charlotte, NC, USA); *cis*-sabinene hydrate (analytical standard, ≥97% (GC)) and terpinene-4-ol (sum of enantiomers, analytical standard, ≥95% (GC)) from Sigma-Aldrich (Darmstadt, Germany).

2.3. GC-MS Analysis and Compound Identification

The chemical composition of the EOs was determined using a Shimadzu GC/MS-Q2010 PLUS chromatograph (Kyoto, Japan), which was interfaced with a Shimadzu GC-MS-QP2010 ULTRA mass spectrometer (Kyoto, Japan). The system was equipped with a non-polar Rxi-5 Sil MS integra guard capillary column (30 m × 0.25 mm × 0.25 µm) from Restek, USA. The analyses were performed in splitless mode, and mass spectra were generated in electron impact mode at 70 eV with a mass range of 33–400 m/z and 0.97 scans per second. Initially, 1 µL of each EO was dissolved in 1 mL of hexane, and then 1 µL of the solution was injected into the GC-MS system.

The GC oven temperature was programmed to start at 50 °C for 1 min, then increased by 5 °C per minute until it reached 160 °C, held at 160 °C for 2 min, and then programmed to increase again to 250 °C at a rate of 10 °C/min. The final temperature was maintained for 4 min. Helium (He) was used as a carrier gas at a flow rate of 1 mL/min. The FID detector and injector temperatures were set at 250 °C, and the ion source temperature was set at 220 °C.

Compounds were identified based on a comparison of experimental retention time (RT) and retention indices (RI) with corresponding published data [3], as well as computer libraries of mass spectra, using "GC/MS solution" v. 2.71 software from Shimadzu and Wiley and NIST. The identification of a compound was approved if mass spectra library data matched computer data with a probability equal to or greater than 90%. The retention indices were calculated relative to the retention times of a series of n-alkanes (C8–C28)

using the GC-MS program described above. The relative percentage composition of the EOs was computed based on GC peak areas without correction factors.

2.4. Behavioural Test

The behavioural assay was performed in a Petri dish using a zone-preferred method as illustrated in Figure 1. The dish was 10.5 cm in diameter and the bottom was covered with filter paper. To one side of the dish, a stimulus consisting of either an EO or a single chemical compound was applied 2–3 mm from the edge. On the opposite side of the dish, a solvent (control) was applied at the same distance from the edge as the stimulus. The EO or chemical compound was applied in five consecutive spots, each containing 2 µL, for a total of 10 µL. The solvent was applied in the same manner and volume. The concentration of the EO was 1 µL/mL in hexane, and a dose of 0.01 µL was used based on preliminary observations. For testing the effects caused by individual compounds, three doses of each were used: 0.01 mM, 0.1 mM, and 1 mM. Hexane was used as the solvent and served as a control. After presenting the stimulus for approximately 0.5 min to allow for solvent evaporation, a single mealworm larva was placed at the centre of the dish. The dish was then positioned under a dim light source, and an air exhaust was adjusted to prevent the accumulation of volatiles. The test was conducted at a temperature of 23 ± 2 °C.

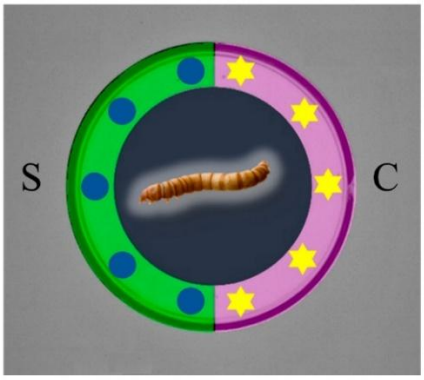

Figure 1. Schematic diagram of the two-choice assay in a Petri dish. The green strip indicates stimulus zone (S), the light violet strip indicates control zone (C); blue circles indicate spots where either EO or single chemical compound was applied; yellow stars indicate spots of solvent drops.

The larva's behaviour was recorded for five minutes using a video camera, and the footage was analysed with EthoVision XT 12, computer-based animal behaviour analysis software developed by Noldus in the Netherlands. Ten behavioural recordings were analysed for each stimulus tested (either EO or chemical compound), and each larva was used only once. The analysis of the mealworm larva's behaviour in both the control and stimulus zones was based on two criteria: distance moved, and time spent in each zone.

2.5. Data Analysis

Statistical analysis of the results was conducted using Microsoft Excel (USA) and STATISTICA (USA) software programs. The distance moved and time spent in the alternative zones were analysed using the Wilcoxon matched-pairs test for both the effects of the EOs and the single compounds. A p-value less than 0.05 was considered statistically significant for the behavioural parameters in the alternative zones.

3. Results

3.1. Movement of Mealworm Larva in the Arena

Movement of mealworm larvae in the arena was recorded, and it was revealed that in the absence of any stimulation, they mostly stayed in the peripheral zone along the edges of the arena, as depicted in Figure 2. Therefore, their behaviour was further recorded only in the periphery of the arena, which was a 1.5 cm wide track referred to as a zone. Prior to testing, we assessed the distance moved by *T. molitor* larvae and the time spent in the left or right zones of the arena. The analysis showed no significant differences (Wilcoxon matched pairs test, $p > 0.05$).

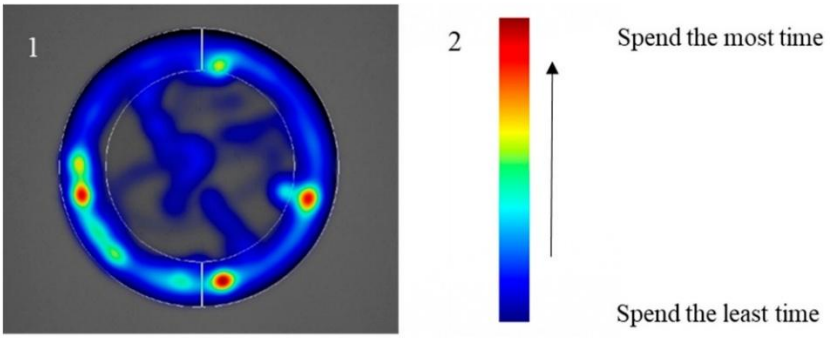

Figure 2. Heat map of *Tenebrio molitor* larvae movement in the arena. **1**—without stimulus (5 min, $n = 10$), **2**—Jet colour scale.

3.2. Effect of Essential Oils

(a) Time spent in zones: While moving in the arena, *T. molitor* larvae spent significantly less time in the zones where either lemongrass, thyme, lavender, clove, or spearmint EO was presented, compared to the time spent in the alternative (control) zone, as shown in Figure 3. The recorded durations differed statistically significantly ($p < 0.05$), and depending on the EO studied, ranged from 4.87 to 15.47 times. However, the eucalyptus EO did not have a significant effect, as the time spent by the larvae in the control and stimulus zones did not differ significantly ($p > 0.05$), as presented in Figure 3. In total, the quantified behavioural data demonstrated that the effect of the studied EOs on the behaviour of *T. molitor* larvae varied from 1.84 (eucalyptus EO) to 5.74 times (peppermint EO).

(b) Distance moved in zones: The distance moved by the larvae in the zone with EO was significantly shorter ($p < 0.05$) compared to the control zone. The distance moved was 2.23 times shorter in the presence of eucalyptus EO, 5.15 times shorter in the presence of lavender EO, 5.29 times shorter in the presence of thyme EO, 6.47 times shorter in the presence of lemongrass EO, 12.76 times shorter in the presence of clove EO, and 17.24 times shorter in the presence of spearmint EO, compared to the control zone, as depicted in Figure 4. Notably, a qualitative change in larval behaviour was observed when exposed to lemongrass EO, as the larvae demonstrated escape behaviour: fell on their side, cringed, and curled.

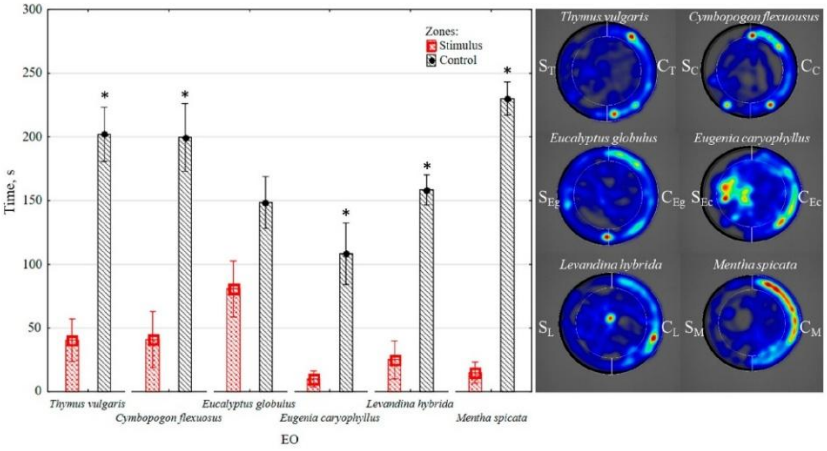

Figure 3. Average time spent by the mealworm (*Tenebrio molitor*) larva in alternative zones: control and containing thyme, lemongrass, eucalyptus, clove, lavender or spearmint EO (5 min, n = 10). Colour on heat maps as indicated in Figure 2. Statistically significant differences of duration in the stimulus and control zones are marked with an asterisk ($p < 0.05$) (Wilcoxon matched pairs test).

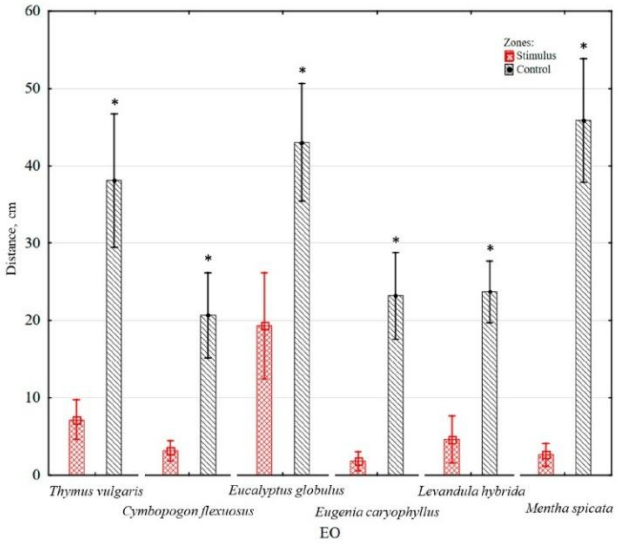

Figure 4. Average distance moved by mealworm (*Tenebrio molitor*) larvae in control and stimulus zone, containing either thyme, lemongrass, eucalyptus, clove, lavender or spearmint EO. Statistically significant differences in the stimulus and control zones are marked with an asterisk ($p < 0.05$) (Wilcoxon matched pairs test).

When summarising the reactions of the mealworms, it should be noted that the effects varied depending on the type of EO used. The effects of each EO were evaluated based on the time spent (t) and distance moved (d) and were classified into three groups: weak effect (if the ratio of S/C changed from 0 to 6), moderate effect (if the ratio changed from >6 to 12), and strong effect (if the ratio changed from >12 to 18). The total effect caused by each EO was evaluated based on both criteria and presented in Table 1.

Table 1. Effect of essential oil on *Tenebrio molitor* larvae behaviour based on the relative time spent and distance moved in stimulus and control zones.

	Distance Moved (d)		Time Spent (t)		(d + t)/2	
EO	S_d/C_d	**Effect**	S_t/C_t	**Effect**	$(S_d/C_d + St/C_t)/2$	**Effect**
Cymbopogon flexuosus	6.47 *	Average	4.87 *	Low	5.67	Low
Thymus vulgaris	5.29 *	Low	4.99 *	Low	5.14	Low
Levandula hybrida	5.15 *	Low	6.35 *	Average	5.75	Low
Eugenia caryophyllus	12.76 *	High	10.85 *	Average	11.80	Average
Mentha spicata	17.24 *	High	15.47 *	High	16.36	High
Eucalyptus globulus	2.23 *	Low	1.84	No	2.04	Low

* statistically significant differences ($p < 0.05$).

The behavioural analysis allows the classification of the EOs tested as having weak, moderate, or strong repelling effects on *T. molitor* larvae. Eucalyptus, lemongrass, thyme, and lavender EOs showed weak repelling effects, while clove EO exhibited moderate repelling effects and spearmint EO was strongly repelling.

To identify the chemical compounds in the EOs responsible for the observed behavioural reactions in *T. molitor* larvae, we conducted an analysis of the chemical composition of the EOs.

3.3. Chemical Composition of EOs

The number and abundance of volatile components identified in the EOs varied depending on the type of EO tested. Lavandin EO contained the highest number of compounds, with 76 detected, while clove and eucalyptus EOs had relatively fewer compounds, with 36 and 28, respectively. Only three compounds, limonene, linalool, and trans-caryophyllene, were common to all six EOs studied, with their abundance ranging from trace to almost 30%. In lavandin EO, linalyl acetate was the most abundant compound. Thyme EO contained *cis*-sabinene hydrate (36.55%) as the main compound, along with terpinen-4-ol (13.08%) and thymol (7.42%), which were absent in the other EOs analysed. Carvone (78.01%) was the major compound in spearmint EO, with a significant amount of limonene (11.31%). Lavandin EO contained linalyl acetate (39.78%), linalool (27.88%), and camphor (8.31%). More than 97% of clove EO was made up of eugenol (75.86%), eugenol acetate (11.64%), and *trans*-caryophyllene (9.84%). The major compound in eucalyptus EO was 1,8-cineole (86.56%), and only three other constituents were found in quantities greater than 1% of the total number of compounds identified: α-pinene (5.22%), γ-terpinene (3.97%), and α-terpineol (1.07%). East-Indian lemongrass EO was rich in geranial (42.07%) and neral (30.48%), with significant amounts of geraniol (6.31%) and geranyl acetate (4.08%). In all the EOs analysed, one or two compounds were predominant only, while others were present in considerably smaller or minor amounts (Table 2).

Table 2. Main composition (% including constituents with quantity above 1.0% at least in one sample) of essential oils.

No	Compound Name	RI_{Exp}/RI_{Lit}	Thymus EO %	Spearmint EO %	Lavandin EO %	Clove EO %	Eucalyptus EO %	Lemongrass EO %
1	*α*-Pinene	939/939	0.95	0.26	0.23		**5.22**	0.12
2	Sabinene	975/975	**2.84**	0.11	0.06		0.09	
3	Myrcene	990/990	**3.75**	0.83	0.32		0.38	0.04
4	*α*-Terpinene	1017/1018	**2.89**				0.16	
5	*para*-Cymene	1024/1024	**6.00**	0.19	0.10		0.72	0.01
6	Limonene	1030/1029	**1.95**	**11.31**	0.50	0.16	tr.	0.24
7	1.8-Cineole	1031/1031	0.28	0.56	**4.15**		**86.56**	
8	*γ*-Terpinene	1059/1059	**6.52**	0.04	0.02		**3.97**	
9	*cis*-Sabinene hydrate	1071/1070	**36.55**	0.40				
10	Allyl hexanoate	1080/1079						**1.28**
11	Terpinolene	1088/1088	**1.02**	0.02	0.04		0.04	0.05
12	Linalool	1096	tr.	0.07	**27.88**	0.02	0.03	**1.18**
13	*trans*-Sabinene hydrate	1098/1099	**5.74**					
14	Camphor	1145/1146	0.15		**8.31**			
15	*cis*-Isocitral	1165/1164						**1.04**
16	Borneol	1169/1169	0.13		**2.39**			0.4
17	Terpinen-4-ol	1177/1177	**13.08**	0.63	**2.59**		0.25	
18	*trans*-Isocitral	1179/1180						**1.87**
19	*α*-Terpineol	1188/1188	**2.36**		**1.09**		**1.07**	0.19
20	*cis*-Dihydro carvone	1193/1192	0.14	**1.05**				
21	Neral	1239/1238					0.03	**30.48**
22	Carvone	1242/1243	0.07	**78.01**			0.03	
23	Linalyl acetate	1252/1257	0.26		**39.78**	0.01		
24	Geraniol	1257/1257			0.18			**6.31**
25	Geranial	1268/1268					0.04	**42.07**
26	Lavandulyl acetate	1289/1290			**3.28**			
27	Thymol	1290/1290	**7.42**					
28	Eugenol	1359/1359				**75.86**		0.04
29	Geranyl acetate	1380/1379			0.58			**4.08**
30	*β*-Bourbonene	1388/1388	0.03	**1.56**	0.03	0.01		
31	*trans*-Caryophyllene	1420/1419	**2.26**	0.80	**1.77**	**9.84**	tr.	**1.51**
33	*α*-Humulene	1454/1454	0.07			**1.09**		0.16
34	*γ*-Cadinene	1512/1513	0.01		0.23			**1.52**
35	Eugenol acetate	1520/1521				**11.64**		

RI_{Exp}: Kovat's indices determined experimentally on the non-polar column Rxi-5 Sil MS integra guard; RI_{Lit}: Kovat's indices for non-polar column Rxi-5 Sil MS integra guard from the literature [3]; tr—trace amount of the compound; relative percentage of compounds above 1% are marked in bold.

The analysis of the chemical composition of the EOs highlighted both predominant and minor compounds. For a more detailed study, compounds of spearmint (the most effective EO) were selected for behavioural testing: both predominant (carvone and limonene) and present in small quantities (myrcene, limonene, γ-terpinene, and cis-sabinene hydrate). Among them, two were predominant in thymus EO (cis-sabinene hydrate and γ-terpinene-4-ol), while the others co-occurred as components of up to 5 EOs analysed/tested (Table 2).

3.4. Effect of Single Compounds

To test the impact of individual chemical compounds, present in the EOs' composition on the behaviour of *T. molitor* larvae, six compounds from spearmint EO (which exhibited high effectiveness) and thyme EO (which belonged to the group of EOs with low activity) were selected.

Carvone. This chemical compound has been found to have an impact on the behaviour of *T. molitor* larvae based on both tested parameters. The larvae moved significantly shorter distances ($p < 0.05$) in the stimulus zone than in the control zone, with a reduction factor of 1.76 at a dose of 0.1 mM and 5.02 at a dose of 1 mM. However, the lowest tested dose of 0.01 mM did not have any effect (see Figure 5A). In addition, the time spent by the larvae in the stimulus zone was significantly changed ($p < 0.05$) only at the highest tested dose of 1 mM, with a reduction factor of 3.62 (see Figure 5B).

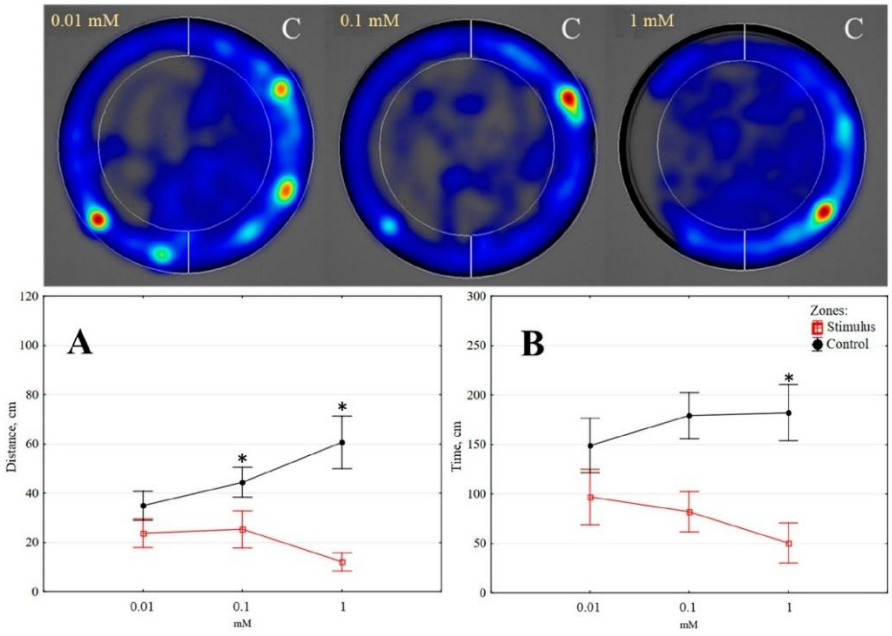

Figure 5. The distance moved (**A**) and time spent (**B**) by *Tenebrio molitor* larvae in alternative zones when exposed to different doses of carvone (5 min, $n = 10$); colour intensity on heat maps as indicated in Figure 2; C—control zone. Statistically significant differences in the stimulus and control zones are marked with an asterisk ($p < 0.05$) (Wilcoxon matched pairs test).

Limonene, myrcene, and γ-terpinene. The distances moved and time spent by the larvae in both the control and stimulus zones were similar compared to the control and did not exhibit significant differences across all tested doses (0.01; 0.1; 1 mM) ($p > 0.05$).

cis-Sabinene hydrate. When evaluating the effect of this chemical compound, a statistically significant difference in activity within the stimulus and control zones was observed only for the distance moved at a dose of 1 mM ($p < 0.05$) (Figure 6A), with the distance in the control zone being 1.92 times longer than in the stimulus zone.

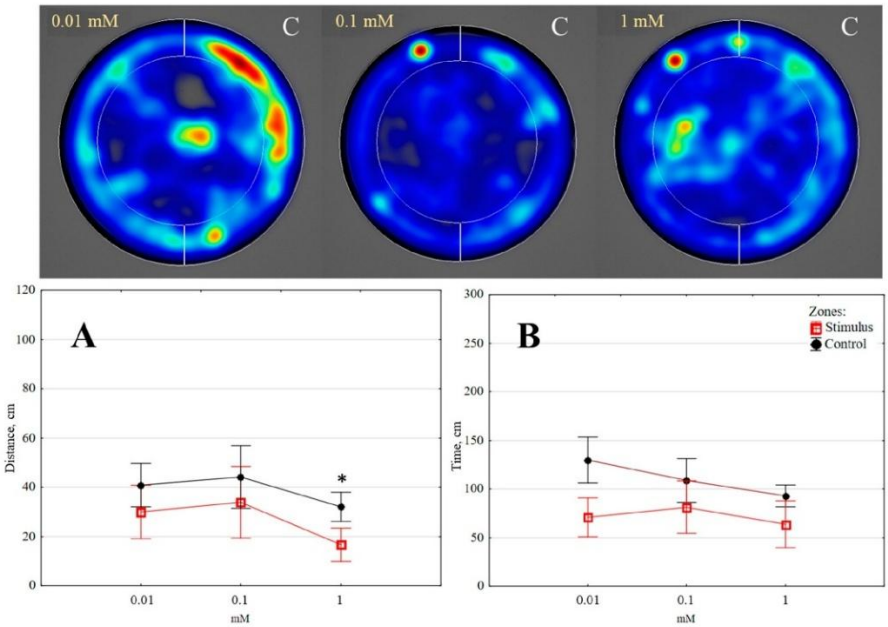

Figure 6. The distance moved (**A**) and time spent (**B**) by *Tenebrio molitor* larvae in the arena after exposure to different doses of *cis*-sabinene hydrate (5 min, $n = 10$); colour intensity on heat maps as indicated in Figure 2; C—control zone. Statistically significant differences in the stimulus and control zones are marked with an asterisk ($p < 0.05$) (Wilcoxon matched pairs test).

Terpinene-4-ol. The larvae's behaviour was significantly impacted by all doses of 4-terpineol tested (0.01 mM, 0.1 mM, and 1 mM) ($p < 0.05$). The distances moved in the control zone were, respectively, 1.81, 3.60, and 2.06 times higher than in the stimulus zone (see Figure 7A).

Although the lowest dose of terpinene-4-ol tested (0.01 mM) did not cause any changes in the time spent in the zones, larvae avoided the zone and spent more time in the control zone by 4.21 and 3.61 times, respectively, when higher doses (0.1 mM and 1 mM) were presented (see Figure 7B). Terpinene-4-ol caused a qualitative change in the behaviour of the larvae, as they fell on their sides, cringed, and curled.

To summarise the larvae's reactions to single chemical compounds tested based on two applied criteria (time spent (t) and distance moved (d) in C and S zones), the compounds were grouped according to their impact. The impact of each tested criterion was classified as low (if the C/S ratio changed from >0 to 2), average (if the ratio changed from >2 to 4), or high (if the ratio changed from >4 to 6). When evaluating the impact according to both criteria, the average value was taken into consideration (see Table 3).

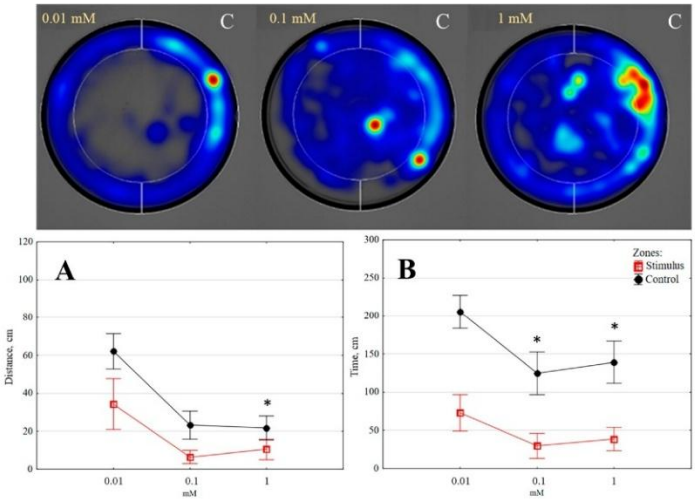

Figure 7. The distance moved (**A**) and time spent (**B**) by *Tenebrio molitor* larvae when exposed to different doses of 4-terpineol (5 min, $n = 10$); colour intensity on heat maps as indicated in Figure 2; C—control zone. Statistically significant differences in the stimulus and control zones are marked with an asterisk ($p < 0.05$) (Wilcoxon matched pairs test).

Table 3. Effect of single compounds on *Tenebrio molitor* larvae behaviour based on the relative time spent and distance moved in stimulus and control zones.

		Distance Moved (d)		Time Spent (t)		(d + t)/2	
Chemical Compound	**Dose (mM)**	C_d/S_d	**Effect**	C_t/S_t	**Effect**	$((C_d/S_d) + (C_t/S_t))/2$	**Effect**
Carvone	0.01	1.47	No	1.54	No	1.51	Low
	0.1	1.76 *	Low	2.18	No	1.97	Low
	1	5.02 *	High	3.62 *	Average	4.32	High
Limonene	0.01	0.85	No	0.77	No	0.81	No
	0.1	1.17	No	1.29	No	1.23	No
	1	1.24	No	1.55	No	1.40	No
Myrcene	0.01	0.98	No	1.21	No	1.10	No
	0.1	1.09	No	1.31	No	1.20	No
	1	0.97	No	0.92	No	0.95	No
γ-Terpinene	0.01	1.13	No	1.18	No	1.155	No
	0.1	1.25	No	1.37	No	1.31	No
	1	0.93	No	1.04	No	0.985	No
cis-Sabinene hydrate	0.01	1.37	No	1.83	No	1.6	No
	0.1	1.30	No	1.34	No	1.32	No
	1	1.92 *	Low	1.46	No	1.69	Low
4-Terpineol	0.01	1.81 *	Low	2.82	No	2.32	Low
	0.1	3.60 *	Average	4.21 *	High	3.91	Average
	1	2.06 *	Average	3.61 *	Average	2.84	Average

* statistically significant difference ($p < 0.05$).

Based on these results, it can be concluded that among the tested chemical compounds present in EOs, terpinene-4-ol has the most significant repellent effect on *T. molitor* larvae, followed by carvone.

4. Discussion

Until now, only the toxic effects of some of the tested EOs on the yellow mealworm, *T. molitor*, have been known, observed either in adults, larvae, or both stages of the beetle. There have been much fewer data on the impact on behaviour, especially at the larval stage.

For instance, topical application of spearmint EO on adult *T. molitor* beetles has been shown to cause toxic effects [4]. Similar effects have been observed in a few species of coleopterans, such as *Epicauta atomaria* (Germar), in both adults and larvae [5]. Our data on the behavioural effect of spearmint EO expand existing knowledge about the impact of this EO on *T. molitor* larvae.

Toxic effects of clove EO on *T. molitor* larvae and adults have been demonstrated by topical application of the EO in acetone on the thorax [6]. Our study expands this knowledge by demonstrating that clove EO is repellent for *T. molitor* larvae, resulting in reduced distance moved in the zone where the EO was presented on the substrate. Moreover, we observed qualitatively different behavioural reactions of the larvae compared to the effects of the other EOs tested, such as dropping, cringing, and curling. Such behaviour can be considered escape behaviour, as reported for larvae of *Drosophila melanogaster* [7]. Our findings on the effect of clove EO on *T. molitor* larvae are novel and provide an opportunity to identify compounds that induce escape behaviour.

The lavandin EO has been previously reported to have a weak repellent effect on *T. molitor* larvae, but this effect was observed after a long exposure period (3 h) only [8]. The method used in the present study allowed much faster detection of this reaction, within 5 min following application. The effect of *Cymbopogon flexuosus* EO on mealworms is unknown. EO of another plant species from the genus Cymbopogon, *C. citratus*, was not found to be repellent to mealworm larvae [9], but it was a strong repellent to another stored grain pest, *Tribolium castaneum* (adults) [10]. In this study, we report novel data on the repellent effect of *C. flexuosus* EO on *T. molitor* larvae, although it was weaker than the effect evoked by the EOs of the other plants studied.

The toxic effect of thyme EO on mealworm larvae and adult beetles has been reported following oil application to wheat feed, but it was not considered one of the strongest-acting oils [11]. The strong toxic effect of thyme EO was observed at very high doses (0.14 mg/cm^3) only [4]. Thyme EO has also been found to have a repellent effect on other adult beetles, including *Sitophilus zeamais* [12], *Meligethes aeneus* [13], and *Ips typographus* [14]. However, the repellent effect of thyme EO on mealworm larvae has not been previously reported.

Previous studies have demonstrated that *Eucalyptus globulus* EO acts as a repellent for adult mealworms [15] but not for larvae [16]. However, dried and ground leaves of this eucalyptus were found to be repellent to larvae. Our results explain the contradiction reported in the study of Martynov et al., (2019): not only leaves but EO of *Eucalyptus globulus* as well is repellent for *T. molitor* larvae [16].

The composition of the EOs used for testing *T. molitor* larvae that caused behavioural reactions was evaluated, as the composition can vary depending on many factors. Thyme (*Thymus vulgaris* L.), a commonly used flavouring agent and medicinal herb, has several chemotypes, including those of linalool, borneol, geraniol, sabinene hydrate, thymol, carvacrol, and multiple other components' chemotypes [17]. The most common chemotypes are thymol, carvacrol, geraniol, and linalool, along with multiple combinations of these compounds. However, borneol and sabinene hydrate chemotypes are considered rare [18,19]. The EO of *Thymus* used in our study belongs to the rare chemotype of sabinene hydrate.

Spearmint EOs are characterised by a high amount of carvone, which can account for over 50% of all identified compounds [20], accompanied by a significant amount of limonene, which can range from 9% to 22% [21]. Hussain et al. (2010) reported that the second most abundant compound in *M. spicata* EO from Pakistan was *cis*-carveol (24%),

with limonene (5.3%) as the third most abundant compound [22]. The EO used in our study had a similar composition of predominant components to that reported by Chauhan et al. (2009) [21].

Lavandin EOs differ from true lavender EOs in terms of much higher amounts of non-major constituents, such as 1.8-cineole (4–7%) and camphor (6–11%). The lavandin EO used in this study, with linalool and linalyl acetate as major compounds, had a standard chemical profile [23].

It has been reported that eugenol, which can account for more than 50% of all identified constituents in clove EO, is usually accompanied by eugenol acetate, trans-caryophyllene, and α-humulene, which can comprise up to 90% of clove EO [24]. The clove EO used in this study contained the same predominant components but was slightly distinguished by the fact that they accounted for as much as 98.4% of all compounds.

The chemical profile of the *Eucalyptus globulus* EO used in this study, with a predominant compound 1.8-cineole and a significant amount of α-pinene, was typical and consistent with the publications [25].

The chemical composition of the lemongrass EO used for testing was similar to the typical composition, with high amounts of neral and geranial as major compounds. These two compounds accounted for over 60% of all identified constituents in the EO [26].

Concerning the data on behavioural effects caused by single chemical compounds from the EOs' bouquet, including carvone, limonene, myrcene, γ-terpinene, terpinene-4-ol, and *cis*-sabinene hydrate, it should be noted that the data are very scarce for many insect species, and for the larvae of *T. molitor*, they were still absent prior to this study.

Carvone has been found to be toxic for some stored grain insect pests at various stages of development, ranging from eggs to adults [27,28]. Caraway (*Carum carvi* L.) EO, which contains approximately 68% carvone, has been shown to be toxic to *T. molitor* adults [29]. In the current study, we identified the repellent property of carvone, which was rated between average and high when compared to other repellent compounds (Table 3).

It is well known that terpinene-4-ol is repellent for adults of the lesser grain borer (*Rhyzopertha dominica*) [30]. This study reports for the first time on the repellent properties of this compound to *T. molitor* larvae, including its ability to evoke a special kind of behavioural reaction called escape behaviour. Interestingly, this compound is quite abundant in thyme EO (13.08%, Table 2), which did not cause behavioural reactions in the larvae (Table 1). Therefore, one can assume that this EO contains compounds that mask the repellent property of terpinene-4-ol.

The effect of *cis*-sabinene hydrate on *T. molitor* larvae behaviour was not known. However, there were reports on the toxic properties of EOs of *Origanum onites* and *O. vulgare* ssp. *hirtum*, which contain a high concentration of this compound (about 14.6%), to adults of the confused flour beetle (*Tribolium confusum*) and the lesser grain borer (*R. dominica*) [31]. As a potentially toxic but low-repellence compound, *cis*-sabinene may be promising for *Tenebrio* larvae control.

The effect of limonene on *T. molitor* larvae has not been studied to date. Moreover, there are no data on its toxicity, although lemon EO, which is rich in this compound is toxic to *T. molitor* larvae [9].

The data we have obtained indicate that γ-terpinene does not cause significant behavioural reactions in *T. molitor* larvae, which is consistent with the weak repellence of bergamot orange EO rich in this compound [8].

Similarly, the effect of myrcene on the behaviour of *T. molitor* larvae was not significant. However, myrcene is one of the major components of *Pistacia atlantica* ssp. *kurdica* EO, which has insecticidal activity against several species of *Tribolium* beetles when used as a fumigant [32]. Therefore, if myrcene were found to be toxic to *T. molitor* larvae (as well as other compounds with similar properties, i.e., limonene, γ-terpinene), it could be a promising agent for pest control purposes, as a toxic compound that does not cause avoidance reactions can be effective at low concentrations.

In summary, the results obtained regarding the behavioural effects of EOs from six plant species and six single chemical compounds present in their composition, which

were tested on *T. molitor* larvae, could be useful for the search and development of non-insecticidal and environmentally friendly means for grain protection. Moreover, these results could be beneficial in the cultivation of *T. molitor* for feed or food, where the control of larval behaviour is needed.

5. Conclusions

Overall, these results highlight that the most effective repellent for mealworm larvae was the EO of spearmint and that of clove showed moderate activity. The EOs of lemongrass thymus, lavandin, and especially EO of eucalyptus were the least repellent. Six compounds of the spearmint EO were tested. The most effective single compounds were terpinene-4-ol and carvone, limonene, myrcene and γ-terpinene were low-effective *cis*-sabinene hydrates and had no significant activity.

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PAPER II

Seed Fatty Acids Modify Oviposition of *Tenebrio molitor* (Coleoptera: Tenebrionidae)

Bumbulytė, G., Auškalnis, A. and Būda, V.

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Seed Fatty Acids Modify Oviposition of *Tenebrio molitor* (Coleoptera: Tenebrionidae)

Gabrielė Bumbulytė [1,*], Arijus Auškalnis [1,2] and Vincas Būda [1]

[1] Laboratory of Chemical and Behavioural Ecology, Nature Research Centre, Akademijos Str. 2, LT-08412 Vilnius, Lithuania; arijusaus@gmail.com (A.A.); vincas.buda@gamtc.lt (V.B.)
[2] Life Sciences Center, Vilnius University, Saulėtekio al. 7, LT-10223 Vilnius, Lithuania
* Correspondence: gabriele.bumbulyte@gamtc.lt

Abstract: Plant–insect interactions mediated by chemical compounds are well documented in roots and above-ground plant parts except seeds. The latter chemoecological interactions remain poorly studied. The chemical composition of seeds, including attractive, repellent, or inhibitory compounds, likely influences oviposition behavior, yet specific studies on this subject are scarce. This study evaluated the oviposition behavior of the yellow mealworm (*Tenebrio molitor* L. (Coleoptera, Tenebrionidae)) on substrates of common oat (*Avena sativa* L. (Poales: Poaceae)), common wheat (*Triticum aestivum* L. (Poales: Poaceae)), rapeseed (*Brassica napus* L. (Brassicales: Brassicaceae)), and pure sand. Females laid the most eggs on oat and wheat substrates, while oviposition on sand was reduced by 22%. The chemoreceptors located in the antennae of *T. molitor* were found not to influence oviposition. Hexane extracts of oat flour were found to contain oviposition-inhibiting compounds, identified as fatty acids. Behavioral tests showed that oleic, palmitic, linoleic, and stearic acids inhibited oviposition at concentrations ranging from 5% to 0.5%. A lower concentration (0.05%) did not have this effect. Additionally, linoleic, palmitic, and oleic acids exhibited repellent properties, whereas stearic acid did not. These findings provide valuable insights into optimizing substrate composition to enhance *T. molitor* reproduction. This has applications for small-scale laboratory research and large-scale industrial production, supporting the use of *T. molitor* as an alternative protein source for feed and food.

Keywords: inhibitor; linoleic; oleic acid; palmitic; repellent; stearic

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1. Introduction

Plant–insect interactions mediated by chemical compounds are well studied in roots and above-ground plant parts [1], playing critical roles in plant and insect life cycles. The exception is chemoecological interactions involving seeds. Those remain underexplored, despite their importance for fundamental understanding and practical applications.

Insects are well known for their significant roles in seed predation [2] and dispersal [3]. In rare cases, they even enhance seed germination. For example, the cotton seed bug, *Oxycarenus luctuosus* Montrouzier (Hemiptera: Lygaeidae), enhances *Gossypium* (Malvales: Malvaceae) seed germination by breaking impermeable seed coats [4]. Some plant seeds possess defensive chemical compounds, which can be either toxic or repellent to insects, deterring herbivory. A notable example is the common bean, *Phaseolus vulgaris* L. (Fabales: Fabaceae), which exhibits resistance to the cowpea seed beetle, *Callosobruchus maculatus* Fabricius (Coleoptera: Chrysomelidae), through seed coat repellency [5].

The mechanisms guiding insect seed recognition for oviposition or feeding remain poorly understood. Seed chemistry, including attractive, repellent, or inhibitory com-

pounds, likely plays a critical role. For instance, ackee (*Blighia sapida* K.D.Koenig (Sapindales: Sapindaceae)) seed extracts reduce oviposition in multiple pests, but the active compounds remain unknown [6]. Similarly, repellency in common bean seeds against *C. maculatus* lacks identification [5].

Seeds of cultivated plants, along with their insect pests, offer suitable models for studying seed–insect interactions mediated by chemical compounds. In this study, we focused on common wheat, *Triticum aestivum* L. (Poales: Poaceae); common oat, *Avena sativa* L. (Poales: Poaceae); and rapeseed, *Brassica napus* L. (Brassicales: Brassicaceae), as seed models. The seed-feeding beetle *Tenebrio molitor* L. (Coleoptera, Tenebrionidae), commonly known as the yellow mealworm, was selected as the insect model due to its widespread occurrence as a stored-product pest and its economic importance globally. While *T. molitor* is primarily considered a pest of stored grains and grain products, it is omnivorous in its natural environment [7]. Furthermore, this species has recently gained attention for its potential as a source of environmentally sustainable protein and as an alternative feed and food product.

The objective of this research was to identify chemical compounds within the seeds of rapeseed, common wheat, and common oat that affect the oviposition behavior of the seed-feeding insect *T. molitor*. Such findings could provide valuable insights not only for fundamental ecological studies but also for optimizing insect rearing under laboratory and industrial conditions.

2. Results

2.1. Oviposition on Different Substrates

The effect of different substrates (oat flour, wheat flour, rapeseed flour, and wheat bran) on *Tenebrio molitor* (with intact antennae) egg laying was significant ($F(56.02) = 3$, $p < 0.001$) (ANOVA). Females with intact antennae laid significantly more eggs ($p < 0.05$) on oat flour and wheat flour compared both to rapeseed flour and wheat bran (Figure 1).

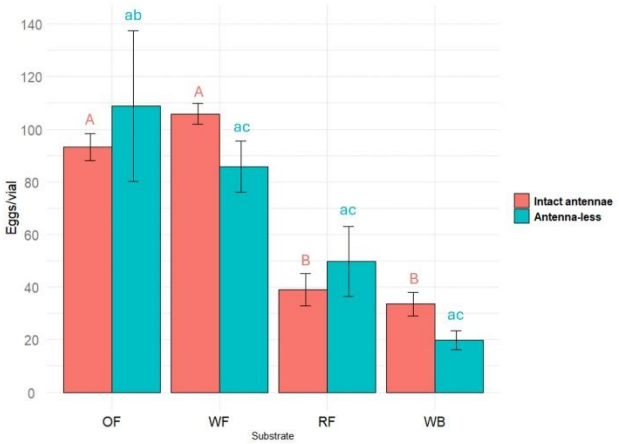

Figure 1. Number of eggs laid by *Tenebrio molitor* females with intact antennae and antenna-less females on different flour substrates: oats (OF), wheat (WF), rapeseed (RF), and wheat bran (WB). Results were analyzed using a one-way ANOVA followed by Tukey's test. Significant differences are indicated by different letters (uppercase for results from females with intact antennae and lowercase for results from antenna-less females) at $p < 0.05$. Error bars represent the standard error.

2.2. The Substrate VOCs and Oviposition

The amputation of the antennae (i.e., the removal of the most abundant olfactory sensillae) did not cause a very pronounced effect in the oviposition test. But the effect of all different substrates (oat flour, wheat flour, rapeseed flour, and wheat bran) on antenna-less *Tenebrio molitor* egg laying was significant ($F(5.601) = 3$, $p = 0.0229$) (ANOVA).

In the antennae-less female sample, which was of the same size as that of females with intact antennae, the standard error increased (this could be a consequence of some damage caused during the antennaectomy). Due to the higher data scattering, the statistical estimates of the differences were less pronounced, but as in the first case, the lowest number of eggs was laid on the substrate of wheat bran, while substrates of oat and wheat flour remained in the group of most suitable for oviposition (Figure 1).

The results obtained indicate that the VOCs of the substrates perceived by olfactory sensillae localized in the antennae of *T. molitor* females did not modulate their oviposition behavior.

2.3. Oviposition on Sand

For the search for biologically active compounds that influence oviposition, it makes sense to have a control substrate without organic compounds but with other properties suitable for egg laying. We chose sand as a possible candidate for such a substrate. It is well known; its composition consists of minerals, with the main component being silicon dioxide (SiO_2).

The effect of different substrates (wheat flour and sand) and antenna being (intact or antenna-less) on *Tenebrio molitor* egg laying was significant ($F(5.203) = 3$, $p = 0.0236$) (ANOVA). In the sand substrate, the females laid about 78% of the number of eggs they lay in their natural wheat flour substrate (Figure 2). A decrease of about 22% was statistically significant; however, it is obvious that this organic-free substrate still can be used for searching for chemical compounds affecting oviposition.

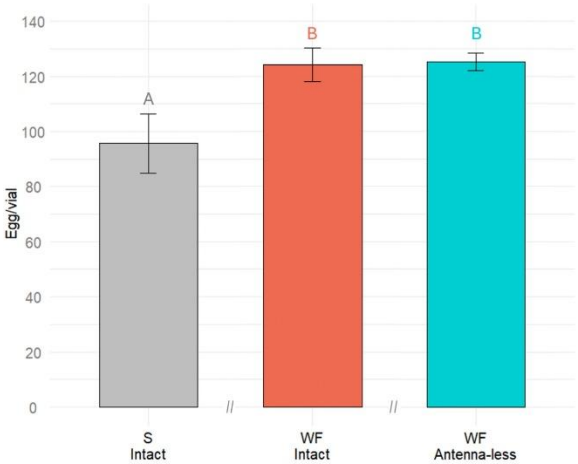

Figure 2. Number of eggs laid by *Tenebrio molitor* intact and antenna-less females to either sand (S) or wheat flour (WF) substrate. Results were analyzed using a one-way ANOVA followed by Tukey's test, with significant differences indicated by different letters ($p < 0.05$). Error bars represent the standard error.

2.4. The Effect of Oat Extract

Data obtained by testing the effects of chemical compounds of oats on oviposition are given in Figure 3. On the sand with oat extract, the females laid just about 4% of the number of eggs compared to on the pure sand. This big difference was statistically significant ($p > 0.05$), and thus the result obtained clearly testifies to the inhibitory effect of the extract.

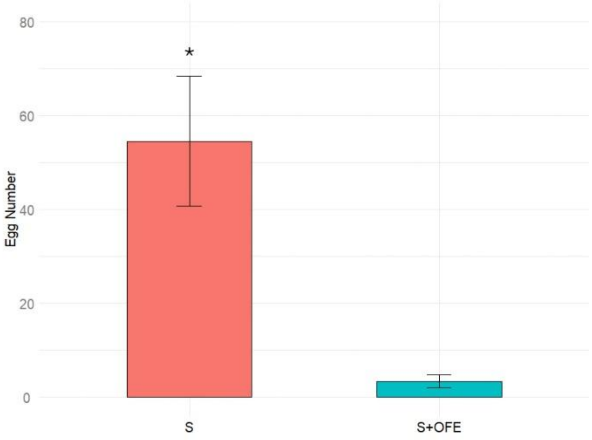

Figure 3. Number of eggs laid by *Tenebrio molitor* females on sand (S) and sand plus oat flour extract (S + OFE). Results were analyzed using a Wilcoxon test, and significant difference with $p < 0.05$ are indicated by asterisk (*). Error bars represent the standard error.

2.5. The Effect of Oat Extract Fractions

To reveal which compounds are present in the extract affect oviposition, fractionation of the extract (complex mixture of compounds) is necessary. We divided the extract into two fractions and tested their effects. For control, oviposition on the substrate of extracted oat was used. In the extract (a mixture consisting of many chemical compounds), some compounds can act as inhibitors and some as stimulators. In this case, the total effect of the mixture is determined by predominant compounds. Among such masked compounds could be both volatile and non-volatile compounds. Therefore, the extract fractions were tested on females both with intact and removed antennae.

The effect of different substrates (oat flour, extracted oat flour, extracted oat flour plus 1st fraction, and extracted oat flour plus the 2nd fraction) on *Tenebrio molitor* (with intact antennae) egg laying was significant $\chi^2(3) = 19.1$, $p < 0.001$ (Friedman test), but the effect on antenna-less *T. molitor* was not significant $\chi^2(3) = 7.4$, $p = 0.06018$ (Friedman test). Females, with antennae, laid more eggs on extracted oat compared to the same substrate plus oat extract fraction (either the first or the second). This difference was statistically significant, $p < 0.05$ (Figure 4). The data obtained reveal that there may be more than one of the compounds that inhibit *T. molitor* female's oviposition and that olfactory stimuli perceived by antennae can be involved in the process of inhibition of the egg-laying.

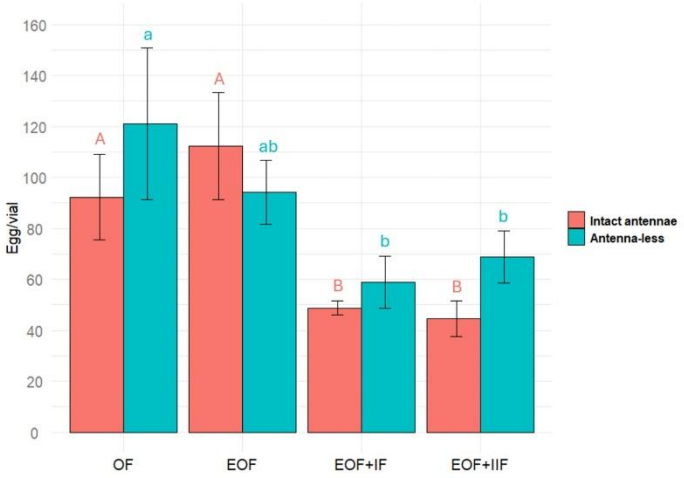

Figure 4. Number of eggs laid by *Tenebrio molitor* female on different substrates (oat flour (OF), extracted oat flour (EOF), extracted oat flour plus 1st fraction (EOF + IF), and extracted oat flour plus 2nd fraction (EOF + IIF)), both intact and antennae-less. Results were analyzed using a Friedman test followed by post hoc Wilcoxon signed-rank tests with Bonferroni correction, with significant differences indicated by different letters ($p < 0.05$). Error bars represent the standard error.

2.6. The Effect of Fatty Acids

Oat extract contained a noticeable layer of fat, so for the study of its biological effect, we looked at the composition, based on already known (published) data. Four of the most abundant fatty acids were selected: linoleic, oleic, palmitic, and stearic, each of which accounts for from 1 to 36% of the fatty acid content of oat grain [8].

In a two-choice test, *T. molitor* females were given the option to lay eggs on either plain sand or sand treated with a fatty acid. The number of eggs laid by the females varied significantly depending on the type of fatty acid and its concentration.

The effect of different fatty acids (stearic, linoleic, palmitic, and oleic) on *T. molitor* egg laying was significant ($F(5.68) = 12$, $p < 0.001$) (ANOVA). When comparing the total number of eggs laid across both options (plain sand and fatty-acid-treated sand), it was found that fatty acids at both 5% and 0.5% concentrations significantly reduced egg-laying overall (Figure 5). This inhibitory effect was observed not only because females avoided laying eggs on the sand with fatty acid but also because they laid fewer eggs on the plain sand option as well (Figure 5). In contrast, the lowest tested concentration (0.05%) of any fatty acid had no such effect.

Besides their inhibitory effect, the repellent feature of linoleic, palmitic, and oleic acids was revealed: this was evidenced by the asymmetry of the egg distribution between the control and the test plates under free choice conditions. A statistically significant effect ($p < 0.05$) was caused by a 5% concentration of oleic and linoleic fatty acids and a concentration of 0.5% of linoleic and palmitic fatty acids (Figure 5).

Neither inhibitory nor repellent effects caused the lowest concentration (0.05%) of the fatty acids tested (Figure 5).

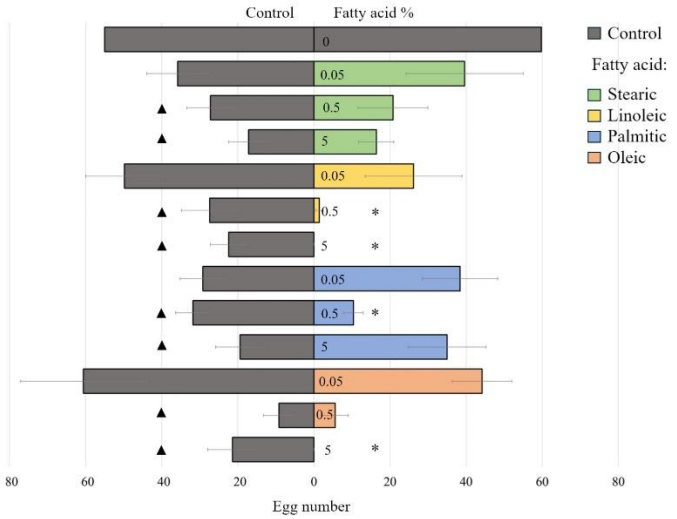

Figure 5. Oviposition of *Tenebrio molitor* females on substrates with four different fatty acids (linoleic, oleic, palmitic, and stearic) and control plates in two-choice test. Tested concentrations: 5; 0.5; and 0.05%. Statistically significant difference ($p < 0.05$) between egg counts on the stimulus and control plates within the same box was marked by asterisk (Wilcoxon test); the same significance in egg counts between the fatty acid test and control test was marked by triangles (one-way ANOVA, followed by Tukey's test). Error bars represent the standard error.

3. Discussion

While the effects of crate size, oviposition duration, adult density, and cannibalism on the reproductive performance of *T. molitor* have been well documented [9], there is a notable gap in understanding the impact of chemical substances in the feed substrate on oviposition. These include stimulatory, inhibitory, deterrent, and repellent effects caused by substrate compounds on the females of this species.

It is known that *T. molitor* females avoid poor-quality food when selecting oviposition sites and usually lay fewer eggs and die sooner when poor-quality food is present [10]. The present study demonstrated that even among high-quality substrates, females oviposit differently. Females exhibited a strong preference for certain substrates, laying the most eggs on oat flour, followed by wheat flour, and significantly fewer eggs on rapeseed flour and wheat bran. This finding highlights oat flour as one of the most favorable substrates for oviposition, likely due to its advantageous physical and chemical properties.

For some insect species, such as the European grape worm, *Lobesia botrana* Denis and Schiffermüller (Lepidoptera: Tortricidae) [11], and the oriental fruit fly, *Bactrocera dorsalis* Hendel (Diptera: Tephritidae) [12], olfactory stimuli are important for oviposition, while for others, like the tobacco hawk moth, *Manduca sexta* L., (Lepidoptera: Sphingidae) [13], they are not. In the case of *B. dorsalis*, it has been established that olfactory receptors associated with oviposition are localized in the antennae [12]. Data on egg-laying regulation in coleopterans, such as the spotted lady beetle, *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae) [14–16], are scarce, and the involvement of antennal chemoreceptors has not yet been analyzed.

The results of the present research clearly demonstrate that *T. molitor* females' antennal chemoreceptors are not involved in the perception of chemical compounds influencing oviposition. The result of our study was obtained in a situation when the phase of the search for a substrate from a distance was eliminated (i.e., the phase when the olfaction and olfactory receptors, localized mostly on the antennae, would have been needed), as the insects and the substrates for oviposition were already at a close range (in small cages).

Before testing the role of chemical compounds in egg-laying, the suitability of a sand substrate that does not contain any organic compounds was assessed. It was found that *T. molitor* females lay their eggs to pure sand substrate, but with lower intensity (approximately 25% less) compared to wheat flour. For future studies, it would be beneficial to include a control group with antennae removed and ovipositing on the neutral/control substrate (sand) to better understand the role of antennal chemoreception in oviposition behavior.

Testing the sand substrate with an oat extract resulted in a significant inhibition of oviposition. This result indicates that chemical cues within the substrate play a crucial role in *T. molitor* females' oviposition behavior. It suggests that the extract contains compounds that suppress oviposition.

The hexane oat extract, when left undisturbed, separated into two layers, with the upper layer undoubtedly consisting of fats. Since the chemical composition of oat fatty acids is known (as well as that of other plant seeds tested; see Table 1), we proceeded to test the most abundant components.

Table 1. Fat content and fatty acid composition of substrates (oat, wheat, rapeseed, and wheat bran).

		Fatty Acids %			
Substrate	**Fat Amount %**	**Oleic**	**Palmitic**	**Linoleic**	**Stearic**
Oat [8,17–19]	4–10	34.80	18.90	38.30	2.10
Wheat [20]	2–4	11.80	21.10	58.30	3.10
Rapeseed [21,22]	40–45	62.25	4.70	17.19	1.80
Wheat bran [23,24]	3–4	14.60	17.70	54.60	2.60

Kouřimská et al., 2018 [8]; Sterna et al., 2014 [17]; Leonova et al., 2008 [18]; Sahasrabudhe, 1979 [19]; Lidon et al., 2019 [20]; Chew, 2020 [21]; Waheed et al., 2013 [22]; Apprich et al., 2014 [23]; and Jung et al., 2010 [24].

Each of the fatty acids tested (oleic, palmitic, linoleic, and stearic) at concentrations ranging from 5% to 0.5% inhibited oviposition in *T. molitor* females. A lower concentration (0.05%) did not have this effect. Very little is known about the impact of fatty acids on the oviposition of other insect species. In spotted wing drosophila, *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), caprylic and capric acids were identified as key components responsible for oviposition deterrence, while the other fatty acids tested had no significant effect [25]. Fatty acids were also found to be important cues for oviposition in the black soldier fly, *Hermetia illucens* L. (Diptera: Stratiomyidae): decanoic acid is repulsive, while tetradecanoic acid is attractive to females and stimulates oviposition [26]. In *Ostrinia latipennis* Warren (Lepidoptera: Crambidae), fatty acids with C_9–C_{22} stimulate oviposition [27]. A mixture of fatty acids stimulates oviposition in the cowpea weevil, *Callosobruchus maculatus*, but elevated levels of oleic acid act as a deterrent [28]. A blend of unsaturated fatty acids decreases oviposition in the pepper weevil, *Anthonomus eugenii* Cano (Coleoptera: Curculionidae) [29]. The effect of fatty acids on oviposition in the oriental fruit fly, *Bactrocera dorsalis*, was more complex: four fatty acids (caprylic, capric, oleic, and linoleic acids) reduced oviposition, two (lauric and myristic acids) had no effect, and two (palmitic and stearic acids) stimulated oviposition [30]. A similar complex effect was observed in another fruit fly species, the melon fly, *Zeugodacus cucurbitae* Coquillett

(Diptera: Tephritidae) [31]. These examples demonstrate that the effect of fatty acids on oviposition is ambiguous and species-dependent, influenced by the organism's biological peculiarities. To date, there have been no reports on the inhibitory effect of fatty acids on *T. molitor* beetles.

In addition to the inhibitory effects, repellent properties were observed for linoleic, palmitic, and oleic acids, while stearic acid did not exhibit this effect. The dual effects of the same fatty acid on the same organism are known [31], though such cases are extremely rare, to our knowledge.

Since both inhibitory and repellent effects were recorded in our tests at concentrations ranging from 5% to 0.5% (but not at 0.05%), a reasonable question arises about the relevance of these findings to real-world conditions. The answer can be found by reviewing previously published data on the qualitative and quantitative composition of fatty acids in grains, as presented in Table 1. These data suggest that *T. molitor* may encounter concentrations of oleic, palmitic, and linoleic fatty acids significantly higher than 5% in natural substrates. The results reported in this paper can help optimize media composition for *T. molitor* reproduction, both for small-scale laboratory research and large-scale industrial purposes. For the most successful reproduction of *T. molitor,* the medium within oviposition chambers must contain less than 5 percent of each of the following fatty acids: oleic, palmitic, and linoleic.

4. Materials and Methods

4.1. Insects

Yellow mealworms (*Tenebrio molitor* L.) were reared in the Laboratory of Chemical and Behavioural Ecology, Nature Research Centre Vilnius, Lithuania on a diet of wheat bran supplied with carrot pieces. To obtain virgin adults, pupae were sexed based on the morphological characteristics of the terminal segments. The pupae were allowed to undergo the final stages of metamorphosis in plastic containers, so that males and females hatched separately. Upon hatching, the adult insects were maintained for 7 days under controlled environmental conditions: a temperature of 25 °C, 60% relative humidity, and a 12:12 h light-dark cycle.

Before mating, male beetles were marked with a green dot (Acrylic marker SCHNEIDER Paint-it 310, 2 mm, green, Wernigerode, Hartz, Germany) on the dorsal side of the thorax to distinguish them from females. The beetles were allowed to mate for 5 days under the same controlled conditions as indicated above and were used in the tests. For olfactory blocking, the antennae were removed at the very base using surgical scissors.

4.2. Oviposition Substrates

Therefore, for biologically active compounds, we chose the following as substrates: oat flakes produced by "Dobele" (Latvia), wheat flour by "Kauno grūdai" (Lithuania), rapeseed flour by "EKKO" (Lithuania), and wheat bran by "Malsena" (Lithuania). Oat flakes were ground into flour and sieved through a 1 mm sieve. The quartz sand was 0.0–0.4 mm fraction produced by "Anykščių kvarcas" (Lithuania). Before use, the sand was washed and heated at 150 °C for 2 h. Solvent hexane was used (purchased from Honeywell, Seelze, Offenbach, Germany, purity $\geq$ 99%).

4.3. Fatty Acids

All fatty acids were bought from Thermo Fisher Scientific (Vilnius, Lithuania) and were of analytical grade: oleic acid ($\geq$98.5% purity, GC), linoleic acid ($\geq$98.5% purity, GC), palmitic acid ($\geq$97.5% purity, GC), and stearic acid ($\geq$97.5% purity, GC).

4.4. Oviposition Test

During the test, there was a box (36 × 26 × 11 cm) containing a mesh-bottomed inner box (35 × 24 × 10 cm; mesh size 1 mm). Beneath the mesh were plastic Petri dishes (3 cm diameter) with different substrates (20 g) for female oviposition (except in sand substrate test).

A total of 10 male and 10 female (intact or antennae-less) beetles were released onto a mesh-bottomed box. A 10 g piece of carrot was added as water source. It was maintained at 25 °C and 60% relative humidity with a 12:12 h light-dark cycle. After 24 h, the substrates from the dishes were sieved, and the eggs were counted. Each test was replicated five times for each substrate.

4.4.1. Difference Between Substrates

For the four-choice test, a Petri dish was supplied with each substrate (oat, wheat, rapeseed flour, and wheat bran). Used female beetles were intact or antennae-less.

4.4.2. Sand Substrate

Beneath the mesh of each inner box, 100 g of one of two different substrates (sand or wheat flour) were placed. Used female beetles were intact or antenna-less.

4.4.3. Oat Extract

For the test, 150 mL of hexane was added to 100 g of ground oats. The mixture was shaken and sonicated in an ultrasonic bath for 480 s, followed by a 1-h extraction period. The settled extract was collected and poured on to 100 g of sand. Beneath the mesh, two Petri dishes were positioned with sand plus oat extract and sand with evaporated solvent.

4.4.4. Oat Extract Fractions

For the test, 150 mL of hexane was added to 100 g of oats in a tightly sealed container. The mixture was thoroughly shaken and sonicated in an ultrasonic bath for 480 s, followed by a 1-h extraction period. The settled extract was then collected and kept in fridge (+5 °C) for 1 h. After this time, the first and second fractions were collected and poured on extracted oat flour. In each four-choice test box, four dishes containing oat flour, extracted oat flour, extracted oat flour plus the first fraction, and extracted oat flour plus the second fraction were placed.

4.4.5. Fatty Acid Analysis

For the test, 30 mL of hexane was poured onto 200 g of the sand, thoroughly mixed, and the solvent was allowed to evaporate. In two control dishes, hexane-treated sand was placed.

Solutions of fatty acids in hexane were prepared by weighing 1 g (5%), 0.1 (0.5%) g, and 0.01 (0.05%) g of each fatty acid and placing them into separate, tightly sealed vials. To each vial, 15 mL of hexane was added, and the mixture was thoroughly shaken. The solutions were sonicated in an ultrasonic bath for 480 s. Each solution was individually poured onto 100 g sand. The solvent was evaporated by mixing until dry mass.

Beneath the mesh, two dishes were positioned: one experimental dish containing the fatty acid-treated sand and one control dish with the solvent-treated sand.

4.5. Statistical Analysis

Statistical analysis of the data was conducted using Microsoft Excel, R version RStudio 2024.09.1+394 (R Core Team, 2023) within the RStudio environment (RStudio Team, 2023). For the analysis of oviposition on different substrates, and oviposition on sand, a one-way ANOVA was applied followed by Tukey's HSD to determine significant differences

between groups. For oviposition in the presence of oat extract fractions, the Friedman test was applied followed by post hoc Wilcoxon signed-rank tests with Bonferroni correction to determine significant differences between groups. For oviposition in the presence of oat extract, the Wilcoxon matched-pair test, and for fatty acid analysis the Wilcoxon matched-pair test and Mann–Whitney U test, were employed. Statistical significance was determined at $p < 0.05$, indicating a confidence level higher than 95%.

Author Contributions: Conceptualization V.B.; formal analysis and investigation, G.B. and A.A.; methodology, V.B. and G.B.; writing—original draft G.B.; and writing—review and editing V.B. All authors have read and agreed to the published version of the manuscript.

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PAPER III

Gut Microbiota and Frass Volatiles: Behavioral Responses of *Tenebrio molitor* and *Tenebrio obscurus* (Coleoptera, Tenebrionidae)

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RESEARCH

Gut microbiota and frass volatiles: behavioral responses of *Tenebrio molitor* and *Tenebrio obscurus*

Vesta Skrodenytė-Arbačiauskienė[1] · Gabrielė Bumbulytė[2] · Arijus Auškalnis[2] · Jorigis Marčėnas[2] · Vincas Būda[2]

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Abstract
Pests in stored grains, *Tenebrio molitor* and *Tenebrio obscurus*, show significant inter and intraspecific as well as sex-specific differences in their gut microbial communities. *T. obscurus* males generally possess higher microbial taxa richness and evenness than females. On the contrary, the gut microbiota of *T. molitor* females exhibits greater diversity and evenness compared to that of conspecific males. Key phyla, such as *Bacillota* and *Pseudomonadota*, are dominant in the gut of both beetle species, with varying relative abundances between the species and sexes. In *T. molitor*, *Spiroplasma* is the most abundant genus in males, whereas *Enterococcus* dominates in females; in *T. obscurus*, *Enterobacter* is the most common genus, whereas *Lactococcus* is more prevalent in females. Behavioral responses of the studied beetles to their own and other species' frass indicated the presence of volatile organic compounds (VOCs) that act as pheromones. Frass of *T. molitor* males contains VOCs that act as an aggregation pheromone, while those of females act as a sex pheromone. VOCs emitted from frass of *T. obscurus* evoked no intraspecific behavioral responses, but those emitted from *T. obscurus* females attracted *T. molitor* males. Differences in gut microbiota composition of closely related beetle species, along with divergent behavioral responses to frass VOCs, may reflect their distinct evolutionary pathways and adaptive specialization for chemical communication.

Keywords Darkling beetles · Yellow mealworm · Gut bacteria · Aggregation pheromone · Sex pheromone

Key message

- *Tenebrio* pests: species- and sex-dependent gut microbiota and the effect of frass VOCs on behavior
- *Spiroplasma* dominates in the guts of *T. molitor* males, while *Enterococcus* is prevalent in females
- *Enterobacter* is most common in *T. obscurus* males, while *Lactococcus* is predominant in females
- *T. molitor* male frass VOCs evoke an aggregation effect
- *T. molitor* female frass VOCs attract *T. molitor* males

Communicated by Christos Athanassiou.

✉ Vesta Skrodenytė-Arbačiauskienė
vesta.skrodenyte@gamtc.lt

[1] Laboratory of Fish Ecology, State Scientific Research Institute Nature Research Centre, Vilnius, Lithuania

[2] Laboratory of Chemical and Behavioral Ecology, State Scientific Research Institute Nature Research Centre, Vilnius, Lithuania

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Introduction

Tenebrio molitor and *Tenebrio obscurus* are species of darkling beetles (Coleoptera, Tenebrionidae) whose larvae, commonly known as mealworms, are pests of economic importance in stored product environments. These insects infest stored grain and food products, leading to substantial economic losses (Rees 2004; Banga et al. 2020). Their larvae are particularly adapted to survive in dry, nutrient-poor conditions, making them resilient pests in storage facilities. *T. molitor* and *T. obscurus* have been reported worldwide (Peng et al. 2019). Using insect pheromones either for mass trapping or for implementation of push–pull strategy is a highly promising technique of integrated pest management, offering an environmentally friendly and effective way both to monitor and control pest populations (Wang et al. 2022).

The insect gut is a unique environment for microbial colonization. The microbial symbionts in the intestinal tract, known as the gut microbiome, are particularly significant for insects as they can profoundly impact the life history, fitness, and competitiveness of their hosts, thereby playing a vital role in ecological and evolutionary processes (Magura

et al. 2024; Wang et al. 2025; Yasika and Shivakumar 2025). Although the insect gut contains relatively few microbial species compared to the mammalian gut, symbiotic bacteria play an essential role in many physiological functions, such as nutrient metabolism, improving nutrient-deficient diets, providing protection against parasites and pathogens, and even regulation hosts' mating and reproduction (Engel and Moran 2013; Fredensborg et al. 2020; Jing et al. 2020; Wang et al. 2023, 2024a). The microbiota of insect guts is also a valuable source of active biomaterials, including volatile compounds involved in intraspecific and interspecific communication. These include pheromones, allomones, kairomones, attractants, and repellents (Davis et al. 2013; Xu et al. 2015, 2016; Ren et al. 2021; Zhou et al. 2025). As chemical communication often requires just small quantities of signaling compounds, even a low number of microbial species may be sufficient to have a significant effect (Dillon et al. 2002; Sharon et al. 2011; Leroy et al. 2011a, b; Engel and Moran 2013). Although knowledge of the taxonomic composition and functional roles of insect microbiomes has increased over the past decade, the specific roles of many gut microbes remain unknown.

Frass, the excreta of insects, plays a crucial role in shaping ecological interactions through microbial communities and chemical signaling. It serves as both a biological substrate and a source of volatile organic compounds (VOCs) that can influence insect behavior, including aggregation and mate attraction (Davis et al. 2013). Despite its significance, the interplay between frass microbiota, chemical emissions, and insect behavioral responses remains poorly understood, particularly in the context of sex-specificity and cross-species interactions. Comparative analysis of microbiota in *T. molitor* and *T. obscurus* was conducted on the larval stage only and reveals both similarities and differences in their microbial communities, which may account for variations in their physiology and behavior (Yang et al. 2021b). Recent advances in metagenomics have provided unprecedented insights into the microbial composition of insect guts and frass, revealing how diet, physiology, and environmental factors shape gut-derived microbial communities (Zhang et al. 2023, 2025). Insects rely on a variety of chemical and microbial cues to navigate their environment, find food, and communicate with conspecifics (Del-Claro et al. 2022; Koufakis et al. 2025). Among these cues, frass can serve as a complex signal containing both nutritional and microbial information (Revadi et al. 2021). Understanding these factors in tandem can shed light on how insects interpret frass cues to navigate their environment and respond to potential competitors.

This study integrates gut microbiota analysis and behavioral assay to investigate how two closely related insect species, *T. molitor* and *T. obscurus*, capable of using the same food resources, interact through their own and each other's frass VOCs' bouquets. The study aims to characterize the microbial abundance and taxonomic diversity in the gut of male and female individuals of both species, and to assess behavioral responses to frass cues in intra and interspecific contexts. By combining these approaches, we aim to deepen our understanding of insect-microbe interactions and their evolutionary adaptive strategies, which determine insect diversity and ecological success.

Materials and methods

Insects

Tenebrio molitor and *T. obscurus* culture was maintained at the Laboratory of Chemical and Behavioral Ecology, State Scientific Research Institute Nature Research Centre, Vilnius, Lithuania, since 2019. The insects were reared on a diet consisting of a mixture of wheat bran (90%), plus dry yeast (10%), and provided with 10 g of carrot slices. Each cohort consisted of 100 ± 5 beetles in a plastic box measuring $40 \times 30 \times 12$ cm. *T. molitor* and *T. obscurus* adults were maintained in separate boxes by species and sex, resulting in a total of four boxes (males and females of each species housed separately). A total of 50 g of feed was provided at the start of the adult period, and no additional feed was added. Carrot slices were supplied once at the beginning. Waste removal and substrate renewal were not performed. During rearing, a temperature of 26 °C, relative humidity of 60%, and a 12:12 h day lamps light–dark cycle was maintained. The beetles were sexed at the pupal stage by examining the ventral side of the last abdominal segment: females exhibit a pair of prominent papillae, whereas males lack these structures (Gerber 1975). Adult beetles aged 10–15 days were selected because *T. molitor* are fully sexually mature and exhibit peak reproductive activity during this period (Gerber 1973; Morales-Ramos et al. 2012). For *T. obscurus*, specific data on the age of sexual maturity or peak activity are not available. To our knowledge, this information has not been reported, and our choice was based on the assumption that closely related species share similar developmental patterns.

Insect preparation

A total of 80 adult beetle specimens, aged 10–15 days (40 T. *molitor* and 40 T. *obscurus*) were randomly selected for gut microbiota analysis. The insects were euthanized by placing them in a freezer (-20 °C) for at least 1 h and dissected immediately after removal. Before dissection, the beetles were sterilized by immersion in 75% ethanol for 1 min, followed by washing three times with sterile distilled water. Each sample consisted of dissected gut tissue with ingested/digested content from five individuals, separately male

(M) and female (F). The gut samples were collected and stored into DNA/RNA Shield Lysis Tubes (Microbe) (Zymo Research Corp, Irvine, CA, USA).

Gut microbiota sequencing

Gut samples were sent to ZymoBIOMICS® Targeted Sequencing Service (Zymo Research, Irvine, CA) for 16S rRNA gene sequencing and bioinformatics analysis.

DNA Extraction Total microbial DNA was extracted using the ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research, Irvine, CA) in accordance with the manufacturer's protocol. A blank negative control and positive mock control ZymoBIOMICS® Microbial Community DNA Standard (Zymo Research, Irvine, CA, USA) were included and processed in parallel with the experimental samples.

Targeted Library Preparation The DNA samples were prepared for targeted sequencing with the Quick-16S™ Plus NGS Library Prep Kit (Zymo Research, Irvine, CA). The primer set used in this study was the 16S rRNA gene Quick-16S™ Primer Set V3-V4 (Zymo Research, Irvine, CA, USA). The sequencing library was prepared using an innovative library preparation process, in which PCR reactions were performed in real-time PCR machines to control the number of cycles and thereby limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned up with the Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA), then quantified with TapeStation® (Agilent Technologies, Santa Clara, CA) and Invitrogen Qubit 1X DSDNA High-Sensitivity Assay Kits ® (Thermo Fisher Scientific, Waltham, WA).

Control Samples The ZymoBIOMICS® Microbial Community DNA Standard (Zymo Research, Irvine, CA) was used as a positive control for each targeted library preparation. Negative controls (i.e., blank extraction control, blank library preparation control) were included to assess the level of bioburden carried out by the wet-lab process.

Sequencing The final library was sequenced on Illumina® NextSeq™ with a P1 reagent kit (600 cycles). The sequencing was performed with 30% PhiX spike-in.

Bioinformatics Analysis. Unique amplicon sequence variants (ASVs) were inferred from raw reads using the DADA2 pipeline (Callahan et al. 2016). Potential sequencing errors and chimeric sequences were also removed with the DADA2 pipeline. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the Zymo Research Database, a 16S database that is internally designed and curated, as reference. Composition visualization, alpha diversity, and beta diversity analyses were performed with Qiime v.1.9.1 (Caporaso et al. 2010). The significance of taxonomic differences in the microbiota between the tested groups was examined using the effect size (LEfSe) algorithm (Segata et al. 2011) with linear discriminant analysis (LDA).

Behavioral test

For the evaluation of the effect caused by frass, a two-choice pitfall test was carried out (Fig. 1). The test was conducted in a 9-cm-diameter plastic Petri dish. Two holes (1 cm in diameter) were cut on opposite sides of the bottom of the dish at equal distances from the edge. The center of each hole was positioned 3.0 cm from the center of the Petri dish, and the distance between the two holes was 6.0 cm, ensuring equal accessibility for both options. Into these holes, 1-cm-diameter plastic tubes with tapered ends were inserted, allowing beetles to pass through into the vials below. The plates were covered with a Petri dish lid that contained approximately 50 small holes (each about 1 mm in diameter) evenly distributed across the center area to allow air circulation during the test. Two 10 mL glass vials were used per Petri dish: one containing 0.1 g wheat bran plus 0.1 g frass (F), and the other containing 0.1 g wheat bran only (control–C). Wheat bran was chosen as a neutral, familiar substrate without frass-derived cues, ensuring that attraction was due to frass-specific signals rather than food-seeking behavior. Each plastic dish was used only once to avoid residual chemical cues, while glass vials were sterilized by dry heat at 180 °C for 2 h before each trial. The plastic tube did not reach the bottom of the glass vial; therefore, once a beetle entered one of the two holes and moved toward the stimulus, it could not return to the Petri dish and made a second choice. Each individual made only one choice per trial.

The beetles were placed in Petri dishes for testing one by one and kept for 24 h at 26 °C, 60% humidity, with a 12:12 day lamps light/dark period. Fifteen replicates were performed for each test. After 24 h, the number of beetles was counted in each pitfall.

Chemical analysis of VOCs

Analysis of VOCs collected from the headspace of *T. molitor* and *T. obscurus* male and female frass was performed using gas chromatography–mass spectrometry (GC-MS). Approximately 3 g of frass were placed into an Erlenmeyer flask, and a precleaned PDMS/DVB 65 µm solid-phase microextraction fiber (SPME) was introduced. VOCs were collected for 2 h, and after its finished, the SPME fiber was immediately transferred to a gas chromatography–mass spectrometry system consisting of a Shimadzu GC-2010 gas chromatograph (GC) coupled with a Shimadzu MS-QP 2010 Plus mass selective detector (MS) (Shimadzu, Kyoto, Japan). The GC was equipped with a non-polar Rxi-5SilMS Integra-Guard column (30×0.25 mm×0.25 µm; Restek, USA), and compound separation was performed under the following

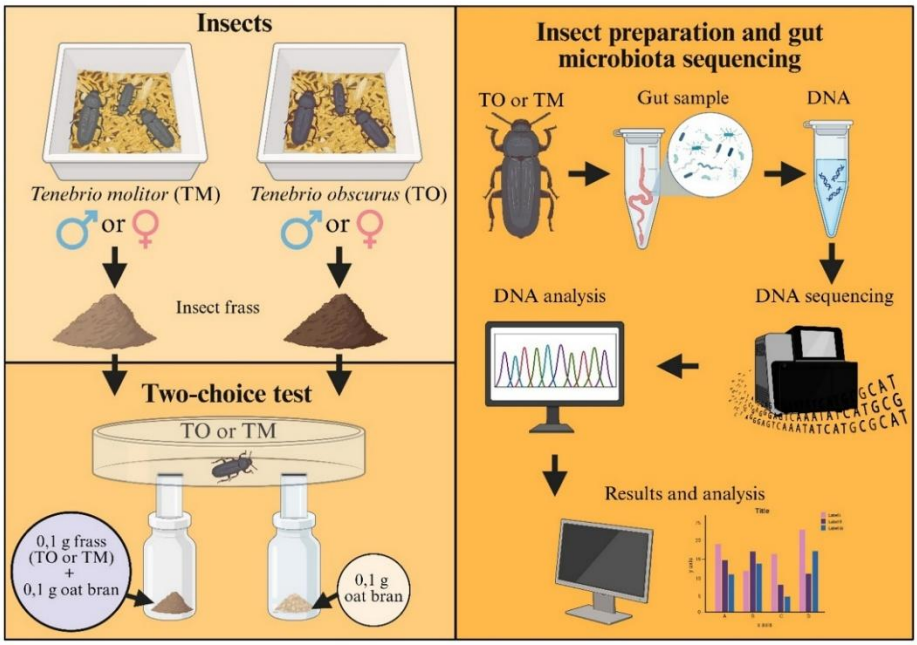

Fig. 1 Preliminary methodological scheme illustrating the main steps of the experimental workflow. Created in BioRender. Tiškevičiūtė, D. (2025) https://BioRender.com/bsqvoqp

conditions: initial temperature 50 °C held for 1 min, then increased to 260 °C at 10 °C/min, and final temperature held isothermally for 12 min. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Electron ionization spectra were acquired at 70 eV. The injector, interface, and ion source temperatures were set at 250, 250, and 200 °C, respectively. MS analysis was performed in scanning mode over a mass range of 33–400 m/z. VOCs were identified by comparing their mass spectra and retention indices with the NIST Mass Spectral Search Program version 14.0 (National Institute of Standards and Technology, Gaithersburg, Maryland, USA). Each sample was analyzed in triplicate.

Statistical analysis

For statistical evaluation, data on alpha diversity were first checked for normal distribution using Kolmogorov–Smirnov and Shapiro–Wilk tests. The normally distributed alpha diversity data were then subjected to one-way analysis of variance (a one-way ANOVA), with results presented as F-statistics. Based on the Bray–Curtis dissimilarity matrix, principal coordinate analysis (PCoA) was performed to compare gut microbiota community compositions among the studied groups. Permutational multivariate analysis of variance (PERMANOVA, with 999 permutations) was used to assess whether there were significant differences in the gut microbiota samples at the amplicon sequence variants (ASVs). PERMANOVA was conducted using PAST (v.4.03). Statistical analysis of the data was carried out using Microsoft Excel and R version RStudio 2024.09.1 + 394 (R Core Team, 2023) within the RStudio environment (RStudio Team, 2023). For the two-choice test with frass, an exact binomial test was applied. Statistical significance was set at $p < 0.05$, indicating a confidence level higher than 95%.

Results

Gut microbiota analysis

Alpha and beta diversity

A total of 4,671,839 high-quality (number of chimera-free sequences that have also undergone further amplicon size filtration) sequences from sixteen gut samples were analyzed (Table S1). Alpha diversity indices summarize microbiota structure based on richness, evenness, or both. Chao1 assesses species richness, Shannon's diversity index evaluates species richness and evenness with greater weight given to taxa richness, and Simpson's index measures species richness and evenness with greater weight given to taxa evenness (Kers and Saccenti 2022). The alpha diversity of the gut microbiota, measured by the Chao1, Shannon, and Simpson indices, revealed significant intraspecific (intersexual) differences between *T. obscurus* males and females. Microbial community richness and evenness were significantly higher in the *T. obscurus* male group compared to the female group (Chao1 $F_{1,6}=19.364$, $p=0.005$, Shannon $F_{1,6}=11.41$, $p=0.015$, Simpson $F_{1,6}=8.07$, $p=0.029$). When comparing the alpha diversity of *T. molitor* between males and females, significant intersexual differences were found for the Shannon and Simpson indices ($F_{1,6}=14.038$, $p=0.009$ and $F_{1,6}=9.946$, $p=0.02$, respectively), while the Chao1 index showed no significant difference. In contrast to *T. obscurus* beetles, the gut microbiota of *T. molitor* females displayed greater diversity and evenness than that of males (Shannon $F_{1,6}=14.038$, $p=0.009$, Simpson $F_{1,6}=9.946$, $p=0.019$) (Fig. 2A, B, C).

This study also revealed significant interspecific differences in gut microbiota between the male beetle species studied. The gut microbiota of *T. obscurus* males was characterized by greater richness, diversity, and evenness than that of *T. molitor* males ($p \leq 0.005$ and $p \leq 0.05$). Meanwhile, the alpha diversity indices of the gut microbiota in *T. obscurus* and *T. molitor* females showed no significant interspecific differences (Fig. 2A, B, C).

The beta diversity of the gut microbiota was assessed using PCoA based on Bray–Curtis dissimilarity distances between the studied groups. The PCoA results revealed apparent intraspecific (intersexual) differences in the gut microbiota of the beetles studied (Fig. 2D). A PERMANOVA (999 permutations) test confirmed significant differences between *T. molitor* males and females ($p=0.024$) and between *T. obscurus* males and females ($p=0.032$). However, no significant differences in microbial community structure were found between *T. molitor* and *T. obscurus* males ($p=0.053$) nor between *T. molitor* and *T. obscurus* females ($p=1.000$) (Table S2).

Taxonomic composition of the bacterial communities

Based on relative abundance, the gut microbiota composition of males and females of *T. molitor* and *T. obscurus* was assessed at different taxonomic levels. Within the gut microbial community of both *T. molitor* and *T. obscurus*, the phyla *Bacillota, Mycoplasmatota,* and *Pseudomonadota* were identified as the most abundant; however, they exhibited different relative abundances across the beetles' gut sample groups under investigation. In *T. molitor* male samples, *Bacillota* and *Mycoplasmatota* were the dominant phyla, representing 52.0% and 43.2% of the total abundance, respectively. Meanwhile, the dominant phylum in *T. molitor* females was *Bacillota,* accounting for 85.8%. The dominant phyla in *T. obscurus* males were *Bacillota* and *Pseudomonadota*, accounting for 50.6% and 46.4% of the total abundance, respectively. Gut samples from *T. obscurus* females, as well as from *T. molitor* females, were dominated by *Bacillota*, accounting for 84.2% of the total (Fig. 3A). At the order level, *Lactobacillales* (51.9%) and *Entomoplasmatales* (43.2%) were dominant in the gut of *T. molitor* males, while *Lactobacillales* absolutely dominated in the females' gut (84.9%). For *T. obscurus*, *Enterobacteriales* (45.2%) were dominant in males, whereas *Lactobacillales* was also most abundant in the female gut microbiota (84.2%), as seen in the *T. molitor* females (Fig. 3B). Bacteria from the *Enterococcaceae* and *Streptococcaceae* families were abundant in the gut of both *T. molitor* and *T. obscurus*, accounting for 18.3 to 51.7%. *Enterobacteriaceae* (45.2%) was the dominant family in the guts of *T. obscurus* males, whereas *Streptococcaceae* (44.6%) dominated in the females' gut microbiota. *Spiroplasmataceae* dominated (43.2%) in the *T. molitor* male gut microbiota, while in the females' gut microbiota, *Enterococcaceae* (51.7%) was dominant (Fig. 3C**,** Table S3).

Analysis of the gut microbiota at the genus level of the studied beetles revealed that the gut microbiota of *T. obscurus* is characterized by higher diversity (20 genera in males and 15 in females) than that of *T. molitor* (12 genera in males and 11 in females) (Table S4). Nine genera were detected in all gut samples from both beetle species and sexes (Fig. S1). The most abundant, as core microbiota, were *Enterococcus* (from 27.72 to 51.70%), *Lactococcus* (from 18.30 to 44.55%), and *Enterobacter* (from 0.13 to 31.60%) (Fig. 3D). *Spiroplasma* was the most abundant genus only in the gut of *T. molitor* males, accounting for 43.2%, while this genus was absent in *T. molitor* females. In the gut of *T. obscurus* males and females, this genus made up only 2.4 and 0.3% of the total abundance, respectively. Additionally, the gut microbiota of *T. obscurus* males showed greater diversity of genera belonging to the family *Enterobacteriaceae* compared to *T. obscurus* females and both sexes of *T. molitor* (Fig. 3D, Table S4).

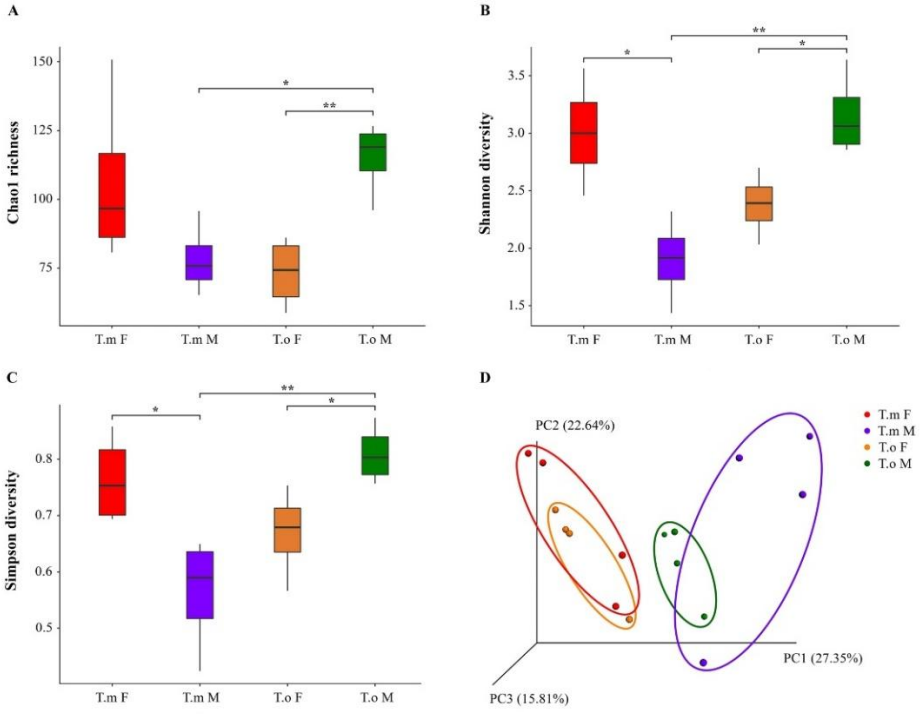

Fig. 2 Comparison of the alpha and beta diversity of the gut microbiota of *Tenebrio molitor* (male–T.m M; female–T.m. F) and *Tenebrio obscurus* (male–T.o M; female–T.o F) among the studied groups. The box plot shows alpha diversity variation across samples: **A** Chao1 richness, **B** Shannon diversity, **C** Simpson diversity. Alpha diversity values are represented as the mean ± standard deviation (SD), $n = 4$. Significant differences between groups $p \leq 0.05$ indicated by * (asterisk), $p \leq 0.005$ indicated by ** (asterisks) (ANOVA). (D) Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity distances between samples at the ASV level

Significant differences in the abundance across taxonomic ranks

LEfSe analysis revealed 13 differentially abundant phylotypes, indicating differences between *T. molitor* males' and females' gut microbiota. In *T. molitor*, six phylotypes were found in males and seven in females (Fig. 4A). For *T. obscurus*, nine phylotypes were identified in males and three in females (Fig. 4B). All these identified phylotypes exhibited high LDA scores (LDA > 2). *T. molitor* males showed a higher abundance of *Spiroplasma* (phylum *Mycoplasmatota*–class *Mollicutes*–order *Entomoplasmatales*–family *Spiroplasmataceae*) (LDA > 5) compared to females. Meanwhile, *Cronobacter–Pantoea* and *Enterobacter* (order *Enterobacteriales*–family *Enterobacteriaceae*), and *Enterococcus* (LDA > 4), were more abundant in *T. molitor* females than in males (Fig. 4A). Significant differences between *T. obscurus* males and females were due to variations in the abundance of *Enterobacter, Lactococcus* spp., *Lactobacillus*, *Enterobacteriaceae* (including *Serratia*, *Citrobacter–Salmonella, Hafnia*, *Klebsiella,* and *Cronobacter–Pantoea* genera), and *Pseudomonas* (LDA > 3) (Fig. 4B).

The LEfSe algorithm also revealed significant differences in some gut microbiota taxa between *T. obscurus* and *T. molitor* males, as well as between females of these species. The gut microbiota of *T. obscurus* males showed a significantly higher abundance of *Enterobacter* (*Pseudomonadota–Gammaproteobacteria–Enterobacteriales–Enterobacteriaceae*) (LDA > 5) and other *Enterobacteriaceae* bacteria (LDA > 3) than that of *T. molitor*

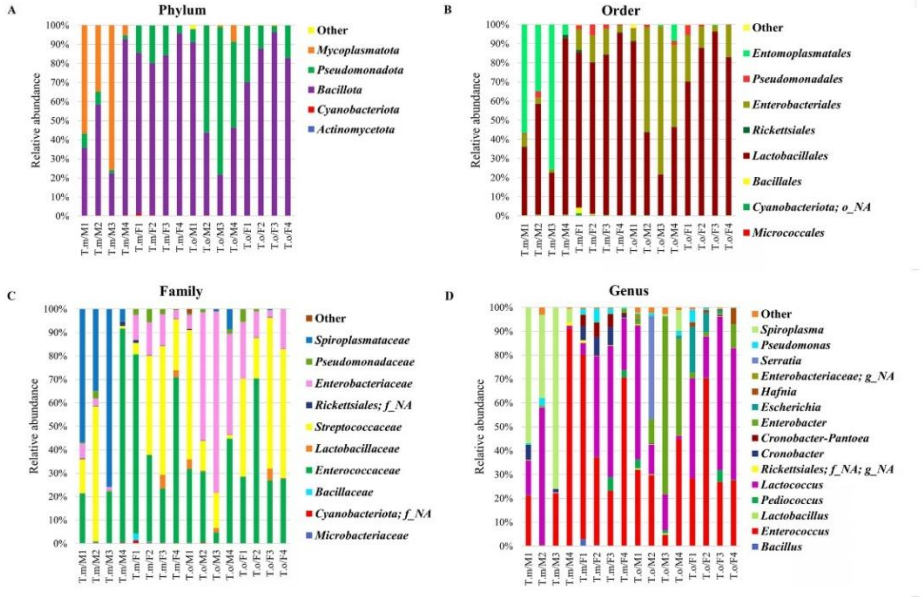

Fig. 3 Taxonomic composition of gut microbiota of *Tenebrio molitor* (male–T.m M; female– T.m. F) and *Tenebrio obscurus* (male– T.o M; female– T.o F) studied groups. Relative abundance of ASVs classified into **A** phylum, **B** order, **C** family, and **D** the 15 most abundant genera. NA – unclassified

males. In contrast, bacteria of the genus *Spiroplasma* (*Mycoplasmatota–Mollicutes–Entomoplasmatales–Spiroplasmataceae*) (LDA > 5) were significantly more abundant in the gut microbiota of *T. molitor* males compared to *T. obscurus* males (Fig. 4C). The gut microbiota of *T. molitor* females comprised 12 phylotypes that were more abundant than those of *T. obscurus* females. Conversely, nine phylotypes were more abundant in *T. obscurus* females than in *T. molitor* females. The most significant differences in abundance between *T. obscurus* and *T. molitor* females were observed for the following phylotypes: *Escherichia*, *Enterobacter*, *Hafnia*, *Enterococcus*, *Cronobacter*, *Cronobacter–Pantoea,* and *Pseudomonas* (LDA > 4) (Fig. 4D). Overall, the results confirm differences in the composition, diversity, and abundance of both interspecific and intraspecific (intersexual) microbial communities.

Behavioral responses to frass

The results revealed sex-dependent and species-specific preferences of frass VOCs. Neither male nor female *T. obscurus* showed a significant response to any of the tested frass sources, regardless of whether the frass originated from conspecifics or heterospecifics, from males or females (Fig. 5).

The behavioral response of *T. molitor* adults to frass differed notably. Males of this species were significantly attracted to frass originating from conspecifics, both males and females, indicating that frass from their own species, regardless of the sex of the insect producing it, contains bioactive compounds that stimulate male attraction. Besides, *T. molitor* males showed a significant attraction to frass from *T. obscurus* females (compared to control), indicating that chemical cues from females of another congeneric species can elicit a cross-species response. *T. molitor* females were attracted to the frass of conspecific males only (Fig. 5).

Chemical analysis of frass VOCs

Preliminary analysis of VOCs' profiles revealed clear qualitative differences between frass from males and females of *T. molitor* as well as *T. obscurus* (Fig. 6). Both species shared some common peaks, but the overall chromatographic

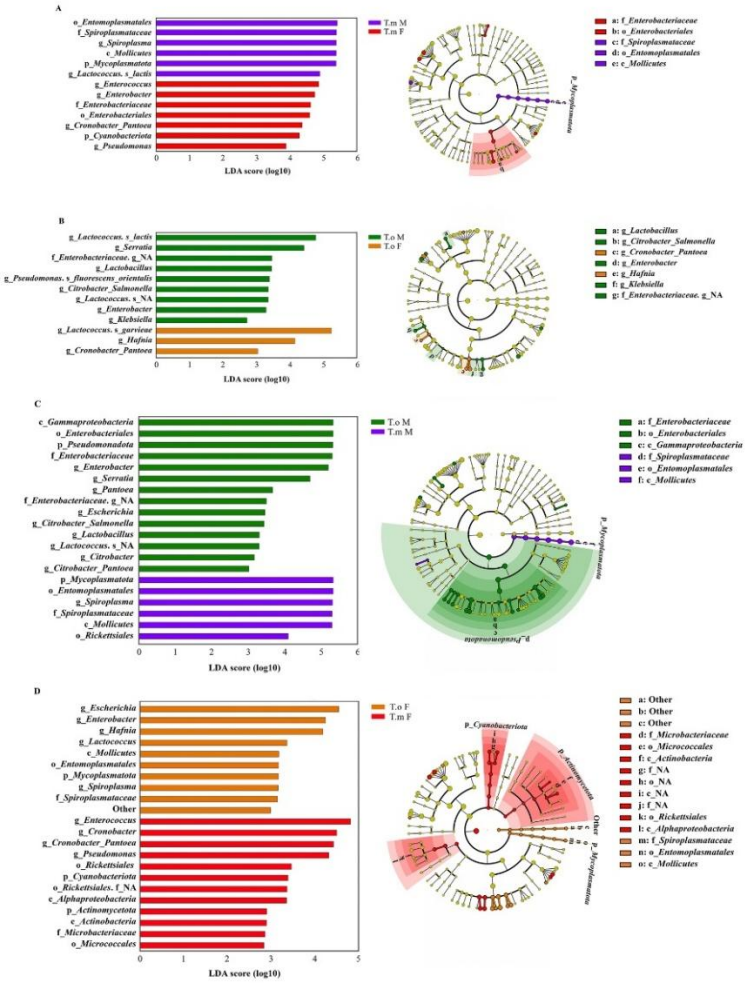

Fig. 4 Linear discriminant analysis effect size (LEfSe) and cladograms showing taxonomic differences in gut microbiota between the **A** *Tenebrio molitor* males and females; **B** *Tenebrio obscurus* males and females; **C** *T. molitor* and *T. obscurus* males; **D** *T. molitor* and *T. obscurus* females. Biomarkers of differently abundant taxa were identified using linear discriminant analysis (LDA) combined with effect size (LEfSe) algorithm. LDA scores indicate the degree of difference in relative abundance at different taxonomic ranks in the tested groups. In cladograms, interconnected circles running from the inside to the outside represent different taxonomic ranks from phylum to genus/species. Each circle represents a distinct microbial taxon at the corresponding taxonomic rank, and the diameter of the circle is proportional to the abundance of the taxon. Taxa with non-significant differences are colored in yellow, while taxa with significant differences are colored according to the colors of the studied groups. NA–unclassified

profiles differed markedly between species and sexes. Due to limitations in the available compound library, individual VOCs were not identified at this stage (low library match factors < 70% and therefore further confirmation required) except two compounds, both of plant origin: terpinolene (retention time 9,61) and trans-caryophyllene (retention time 13,62). However, GC profiles obtained highlight potential chemical cues that may underlie the behavioral differences observed between species.

Discussion

Using next-generation, high-throughput 16S rRNA gene amplicon sequencing, the gut microbiota of *T. molitor* and *T. obscurus* adults, separately males and females, was analyzed for the first time. The microbial community richness and evenness are significantly higher in *T. obscurus* males than in females. In contrast, *T. molitor* females exhibit greater diversity and evenness than males (Fig. 2A, B, C). This finding aligns with other studies, such as the observation that female stag beetles (*Odontolabis fallaciosa*) exhibit higher bacterial alpha diversity (richness and diversity) than males (Wan et al. 2020). Another study also indicated sexual divergence in gut bacterial communities in insects. In this case, the gut microbiota of male field crickets (*Teleogryllus occipitalis*) was more diverse than that of females (Hirata et al. 2023).

Significant interspecific differences in alpha diversity were observed among the males of both species. The gut microbiota of *T. obscurus* males showed greater richness, diversity, and evenness than that of *T. molitor* males ($p \leq 0.005$ for richness and $p \leq 0.05$ for diversity and evenness). In contrast, no significant differences were found in the alpha diversity indices of the gut microbiota from females of these two beetle species (Fig. 2A, B, C). Lower alpha diversity indicates a more specialized and possibly stable gut microbial community, whereas higher diversity suggests a more adaptable microbial ecosystem that can respond flexibly to habitat variability and environmental changes (Mendoza-Guido et al. 2023).

This lack of interspecific variation in adults contrasts with previous studies on the larval stage of these species, which reported significant differences. For instance, the alpha diversity (Shannon and Simpson indices) of gut bacteria in natural wheat bran-fed *T. molitor* larvae was notably higher than in *T. obscurus* larvae (Yang et al. 2021b). The differences in gut microbiota diversity between *T. molitor* and *T. obscurus* larvae and adults may be linked to their different developmental stages and diets. It is well known that variations in insect gut microbiota composition and diversity are strongly associated with their life cycle stages (including larvae, pupae, and adult males and females), feeding habits, and taxonomy (Suárez-Moo et al. 2020; Yasika and Shivakumar 2025). In this study, adult insects were supplied with a controlled diet of a mixture of wheat bran (90%) and dry yeast (10%), supplemented with carrot slices. The results reveal a clear and significant difference in the gut microbial communities of the closely related darkling beetles, *T. molitor* and *T. obscurus*, even though they were both fed the same diet.

In this study, it was found that *Bacillota* (synonym *Firmicutes*), *Mycoplasmatota* (synonym *Tenericutes*), and *Pseudomonadota* (synonym *Proteobacteria*) were the most abundant phyla in the gut microbial community of *T. molitor* and *T. obscurus* adults. *Mycoplasmatota* (o_*Mollicutes*–c_*Entomoplasmatales*–f_*Spiroplasmataceae*–g_*Spiroplasma*) was dominant only in *T. molitor* male gut samples, whereas *Bacillota* (f_*Enterococcaceae*–g_*Enterococcus*; f_*Streptococcaceae*–g_*Lactococcus*) was dominant in female gut microbiota. *Pseudomonadota* (c_*Gammaproteobacteria*–o_*Enterobacteriales*–f_*Enterobacteriaceae*–g_*Enterobacter*) and *Bacillota* (f_*Enterococcaceae*–g_*Enterococcus*) were dominant in *T. obscurus* male gut samples, whereas *Bacillota* (f_*Streptococcaceae*–g_*Lactococcus*; f_*Enterococcaceae*–g_*Enterococcus*) were more abundant in the female gut microbiota (Fig. 3 and 4). The dominant phyla *Bacillota* and *Pseudomonadota* in the insect's gut play key roles in nutritional supplementation, energy absorption, maintaining gut homeostasis, and host immunity, as well as in combating pathogens (Colston and Jackson 2016; Wang et al. 2020; Fredensborg et al. 2020; Fu et al. 2023). The genera *Enterococcus*, *Lactococcus*, and *Enterobacter* were identified as core microbes present in gut samples from both species and sexes of *T. obscurus* and *T. molitor*. These core gut bacteria could be essential for nutrition, physiology, behavior, and defense mechanisms in the tested insects. Previous studies have also identified *Enterococcus* as the most common gut flora in the larval stages of *T. obscurus* and *T. molitor*, and these bacteria are linked to nitrogen metabolism (Jung et al. 2014; Yang et al. 2021b; Ding et al. 2024). *Enterococcus* spp. can also influence the process of insect metamorphosis (Kong et al. 2023). This genus is commonly found in plant-eating insects and has been linked to the ability to metabolize alkaloids (Vilanova et al. 2016). Furthermore, the dominance of *Enterococcus* bacteria in the gut of *Galleria mellonella* larvae has been shown to play an immunomodulatory role in protecting the host from pathogenic bacteria and fungi (Krams et al. 2017; Upfold et al. 2023). It was also found that gut bacteria, including *Enterobacter* (phylum *Pseudomonadota*), are known to confer host resistance against parasitic infections, such as helping in the elimination of tapeworms in *T. molitor* (Fredensborg et al. 2020). This suggests that these predominant taxa make a significant contribution to host fitness and defense mechanisms. The genus *Lactococcus* is relatively abundant in the guts of both larvae and adult insects and

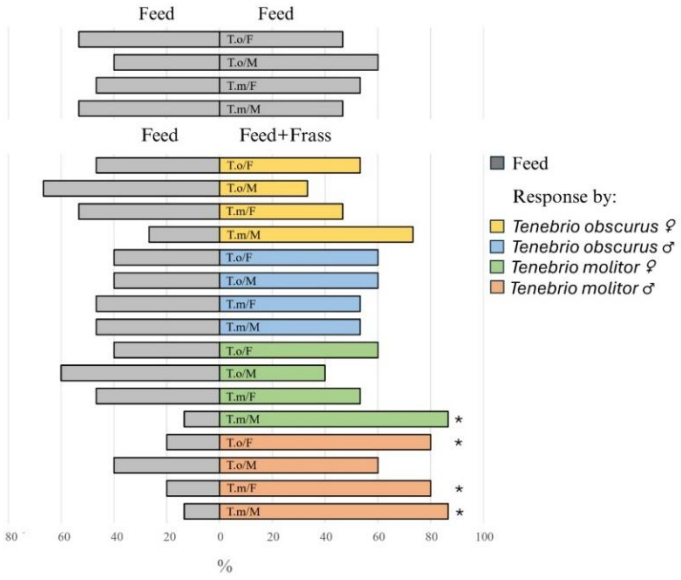

Fig. 5 Behavioral responses of *Tenebrio molitor* and *Tenebrio obscurus* adults to frass' VOCs from the same and different species, as well as the same and different sex. Exact binomial test, significant differences at $p < 0.05$ are indicated by the.* (asterisk)

has pivotal roles in processes such as carbohydrate fermentation and is also capable of producing biotin and Vitamin C (De Jonge et al. 2020; Li et al. 2023; Chen et al. 2024). *Lactococcus* produces lactic acid, which helps regulate gut pH and maintain a balanced microbial community, as well as support intestinal immune protection (Kibet et al. 2024). Additionally, several genera identified in the current study, including *Spiroplasma*, *Enterococcus*, *Lactococcus*, *Enterobacter*, and *Serratia*, have been previously linked to the biodegradation of low-density polyethylene in *T. molitor* and *T. obscurus* larvae (Yang et al. 2014, 2021b; Brandon et al. 2018; Przemieniecki et al. 2019).

Although beta diversity analysis did not reveal significant interspecific differences between *T. molitor* and *T. obscurus* males, or between females, in the gut microbial community, LEfSe analysis showed significant differences in the abundance of specific taxa (Fig. 4). Notably, the *Spiroplasma* genus (family *Spiroplasmataceae*) was only dominant in the gut microbial community of adult *T. molitor* males (43.2%), whereas it was not detected in females. *Spiroplasma T. obscurus* accounted for just 2.4% of male gut microbiota and only 0.1% of female gut microbiota. This highlights the clear species- and sex-specific specialization of insects. The *Spiroplasma* genus is well known for providing host protection against various natural enemies, including nematodes, parasitoid wasps, and fungi (Ballinger and Perlman 2019). It is also recognized as a facultative insect endosymbiont, largely maternally transmitted but capable of occasional horizontal transmission, and potentially involved in reproductive manipulation (Engel and Kaltenpoth 2018; Kakizawa et al. 2022). *Spiroplasma* was also one of the dominant genera found in bran-fed *T. molitor* and *T. obscurus* larval gut samples (Yang et al. 2021b). A detailed phylogenetic analysis revealed that *Spiroplasma* sequences from the guts of *T. molitor* larvae differed from those of previously identified species, indicating the presence of a novel *Spiroplasma* species (Jung et al. 2014). The results of this study suggest that potentially novel species in the *Spiroplasma* community of *T. molitor* larvae, which are transmitted to adult *T. molitor* males and are dominant in the gut, may be functionally significant. In contrast, the gut community of adult *T. obscurus* males is characterized by the predominance of bacteria from the *Enterobacteriaceae* family, which accounts for 45.2% of the total community. Specifically, the intestines of *T. obscurus* males harbored several genera from the *Enterobacteriaceae* family, including *Citrobacter*, *Citrobacter–Pantoea*, *Citrobacter–Salmonella*, *Salmonella*, *Serratia*, and *Pantoea*, which were not detected in *T. molitor* males. This indicates a more complex and potentially more dynamic microbial ecosystem in the gut of *T. obscurus*. For instance, *Serratia*, detected in the *T. obscurus* male community (11%), has been shown in previous studies to have a higher capacity to degrade carbohydrates, including lignocellulose, in the guts of other adult and larval beetles (*Cytrotrachelus buqueti*)

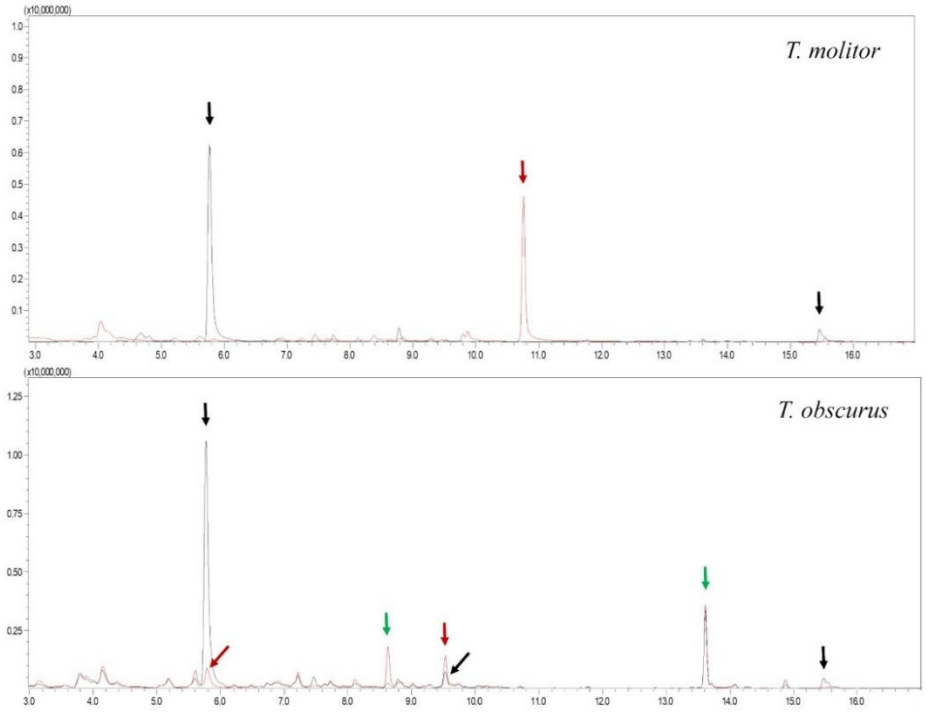

Fig. 6 Gas chromatograms of VOCs from frass of *T. molitor* and *T. obscurus*, produced by males (black line) and females (red line). Black arrows indicate compounds detected exclusively in male frass, red arrows indicate compounds specific to female frass, and green arrows mark compounds identified by MS as originating from plants

(Luo et al. 2019). Furthermore, some symbiotic gut *Serratia* bacteria isolated from beetles have been found to produce the antibacterial cyclic lipopeptide serrawettin W2 and may contribute to controlling bacteria and nematodes in the gut (Heise et al. 2019). While both *T. molitor* and *T. obscurus* exhibited a low overall gut microbiota diversity compared to other beetles (Yun et al. 2014; Ebert et al. 2021), likely due to their narrow dietary specialization, a comparison of the females revealed that the *Enterobacteriaceae* family diversity was notably more abundant in *T. obscurus* than in *T. molitor.*

Gut bacteria are known to produce a wide range of VOCs as part of their metabolic processes. Many of these microbial VOCs can act as semiochemicals, influencing insect behavior (Wang et al. 2023). Previous studies have shown that bacteria from families such as *Enterobacteriaceae*, *Pseudomonadaceae*, and *Bacillaceae* are involved in producing VOCs that serve as pheromones for various insect species (Leroy et al. 2011a, b). Additionally, gut microbes can affect the host's chemical communication by providing or depleting precursor metabolites necessary for the host's own pheromone production. Many insect pheromones originate from basic metabolites, such as fatty acids, terpenoids, and sterols, which can be processed or influenced by gut bacteria (Engl and Kaltenpoth 2018). Wang et al. (2024b) demonstrated in their research that gut bacteria increase male attractiveness to females by promoting the production of the pheromone 4,8-dimethyldecanal (4,8-DMD) in *Tribolium castaneum* (Coleoptera: Tenebrionidae). Other studies have demonstrated that *E. coli* in the gut can indirectly regulate the production of this pheromone in *T. castaneum* by mediating the expression of the *TcFAS1* gene (Zhou et al. 2025). The gut microbial community of the German cockroach (*Blattella germanica*) has also been shown to significantly contribute

to the production of volatile carboxylic acids (VCAs), which function as fecal aggregation pheromones, highlighting the crucial role of gut microbes in insect-insect communication (Wada-Katsumata et al. 2015). Similarly, *Enterobacter agglomerans* (syn. *Pantoea agglomerans*), found in the gut of the Queensland fruit fly (*Bactrocera tryoni*), produced various VOCs, including pyrazines (Tallon et al. 2023). Pyrazines are a chemically diverse class of volatiles that act as multifaceted mediators in insect chemical communication (Silva-Junior et al. 2018; Khashaveh et al. 2025). It was also found that *Pseudomonas putida*, a member of the *Pseudomonas* genus, can produce 2,5-dimethylpyrazine (2,5-DMP), emphasizing the metabolic potential within this bacterial group (Janamatti et al. 2022). In this study, behavioral tests revealed that the frass of *T. molitor* contained VOCs that acted as sex pheromones, depending on the sex producing the frass and as aggregation pheromone in frass of males. Preliminary GS analysis revealed compounds, which can act as these pheromones; however, standard MS libraries were insufficient for reliable identification of these compounds; therefore, further deep analysis is required to identify the individual VOCs, synthesize them, and evaluate their biological activity through behavioral assays. We speculated that these VOCs may originate from microbes. Bacteria from the families *Enterobacteriaceae*, *Pseudomonadaceae*, and *Bacillaceae*, which are known as potential VOC producers, were identified in all tested beetle gut samples. However, only the frass of *T. molitor* males attracted *T. molitor* males and females, but not *T. obscurus* males and females, indicating the presence of aggregation pheromones for this species. It was also found that *T. molitor* female frass attracted males of their own species, indicating the presence of sex pheromones. *T. obscurus* males and females did not respond to either their own or other species' frass. These behavioral differences might be due to significant variations in specific gut bacterial species and their abundance within the beetles' guts. Different gut microbiomes can provide distinct metabolic capabilities. Additionally, insects may respond differently to the same VOCs. A single infochemical can serve multiple functions, and the bioactivity of any individual VOC depends on insect species, life stage, habitat, and environmental factors (Davis et al. 2013). Past research has shown that gut bacteria, such as *Pseudomonas*, *Lactococcus*, *Enterococcus,* and *Rahella*, can produce the multifunctional pheromone verbenone in the gut and frass of the bark beetle *Dendroctonus valens*. This pheromone acts as an attractant at low concentrations and a repellent at high concentrations for *D. valens* (Gillette et al. 2006; Zhang et al. 2006; Xu et al. 2015, 2016). Therefore, the hypothesis proposed in this study–that *T. molitor* gut and frass bacteria release VOCs functioning as aggregation and sex pheromones–warrants further detailed investigation, including the identification of both active compounds and their producers.

The role of frass volatiles in the behavior of adult *T. molitor* and *T. obscurus* has not been previously investigated and remains unclear. Previous work has demonstrated that aggregative behavior is highly expressed in *T. molitor* larvae when exposed to chemical stimuli from the larvae's frass, and Weaver and McFarlane (1989a, 1989b) demonstrated that aqueous solutions of larval frass elicited attraction in late-instar larvae and identified lactic acid as the primary chemical responsible for the response. Significantly, their chemical and behavioral assays indicated that lactic acid acts as a contact aggregation pheromone, detected by substrate probing with mouthpart palpi. In this study, we investigated the behavioral response of adult beetles to frass-emitted volatile cues, without direct contact. Through volatile assays, we established that both adult females and males were attracted to frass from adult males, indicating the presence of an aggregation pheromone in male frass. This provides the first demonstration of *T. molitor* adult attraction to frass volatiles, mediated by VOC(s). Furthermore, our results highlight interspecific differences in chemo communication among closely related species: while *T. molitor* males respond to frass from both sexes of their own species and another species, *T. obscurus* males do not respond to VOCs of any frass (from both conspecific and heterospecific sources, regardless of sex). The revealed difference may reflect diverse strategies in the evolution of chemical communication in closely related species. Future studies will focus on the chemical characterization of the volatiles responsible for these responses with the aim of better understanding the underlying mechanisms of interspecific and intraspecific communication.

Conclusions

This study offers new insights into the biology and chemical ecology of closely related *Tenebrio* species, which are pests in stored grains. Using a new generation of high-throughput sequencing, significant inter and intraspecific (intersexual) differences were identified in the gut microbiota of *T. molitor* and *T. obscurus*, when fed the same diet. The gut microbiota of the studied beetle groups significantly differed in alpha and beta diversity, as well as in various taxonomic groups. Behavioral responses of the *T. molitor* to their frass indicated the presence of chemical compounds that act either as aggregation or sex pheromones, depending on the feces producer's sex. Variations in the gut microbiota composition of the beetles, along with divergent behavioral responses to VOCs, suggest different evolutionary paths and adaptive specialization of closely related beetle species. The results obtained open a new frontier in environmentally friendly pest control

within storage facilities. Further research will focus on identifying both active compounds and their producers.

Author contributions

Conceptualization V.B.; formal analysis and investigation V.S-A., G.B. and A.A.; methodology V.B., V.S-A., J.M. and G.B.; writing–original draft V.S-A. and G.B.; writing-review and editing V.B. All authors have read and approved the final manuscript.

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Data availability Data is provided within the supplementary information files.

Declarations

Conflict of interest The authors declare no competing interests.

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PAPER IV

Yellow mealworm infestation caused by *Hyphopichia burtonii* (Saccharomycetales: Pichiaceae) and associated changes in VOC

Bumbulytė G., Čepukoit D., Būdienė J., Burokienė D., Būda V.

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Yellow mealworm infestation caused by *Hyphopichia burtonii* (Saccharomycetales: Pichiaceae) and associated changes in VOC

Gabrielė Bumbulytė [a,*], Dovilė Čepukoit [b], Jurga Būdienė [a], Daiva Burokienė [b], Vincas Būda [a]

[a] Laboratory of Chemical and Behaviour Ecology, State Research Institute Nature Research Centre, Akademijos Str. 2, LT 08412 Vilnius, Lithuania
[b] Laboratory of Plant Pathology, State Research Institute Nature Research Centre, Akademijos Str. 2, LT 08412 Vilnius, Lithuania

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ABSTRACT

This study provides new insights into yeast-insect interaction and emphasises the potential of yeast as an overlooked factor in insect health. As edible insects become increasingly integral to the global food system, a comprehensive understanding of their microbiology is of paramount importance. Molecular analysis demonstrated that the culture of yellow mealworm, *Tenebrio molitor* (Coleoptera, Tenebrionidae), was infested by the yeast *Hyphopichia burtonii*. No mortality was observed within 7 days of exposure to the yeast, but infestation suppressed locomotory activity to some extent. During the infestation, the scent of volatile organic compounds emitted by the insects changed: the amount of limonene significantly increased. This compound possesses both bactericidal and insecticidal properties. No hazardous compounds for humans were detected. Infected beetles no longer release their sex pheromone. This indicates that the reproductive system is disrupted. *T. molitor* cannot distinguish between infested individuals and healthy ones by smell, which is favourable for the rapid spread of the infestation in the colony.

1. Introduction

As the global demand for sustainable protein sources increases (Nirmal et al., 2025), edible insect rearing has gained increasing attention as an environmentally friendly and nutritious alternative to traditional livestock farming. Due to their high protein content, essential amino acids, and favourable feed conversion (Veldkamp and Bosch, 2015), insects such as *Tenebrio molitor* L. (Coleoptera, Tenebrionidae) are now being mass-reared both for human consumption and animal feed. However, as insect farming scales up, so does the need to know the potential risks associated with the microorganisms that can cause diseases in these insects. The microbial communities associated with edible insects can affect not only their health and development, but also food safety, nutritional quality, and potential biotechnological applications.

Microbial infestation plays a significant role in insect ecology, affecting the physiology, behaviour, and survival. Despite the growing industrial cultivation of *T. molitor*, comprehensive data on its microbial associations are still limited. Some microorganisms are beneficial as symbionts; some are neutral under natural conditions. In mass rearing systems, however, these interactions may become harmful due to high densities and artificial environmental conditions inadequate to natural ones (Slowik et al., 2023). In the context of large-scale insect farming, disease outbreaks caused by entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* (*Hypocreales*), which are widely studied insect pathogens with a broad host range, represent a major challenge to sustainable production (Araújo and Hughes, 2016; Hong et al., 2024). These fungi infect their hosts by penetrating the cuticle with the help of enzymes such as chitinase, leading to systemic colonization and mortality (Vega et al., 2012). In *T. molitor*, infestation is typically characterized by the growth of the fungus on cadavers under humid conditions, followed by the production of conidia – green in *Metarhizium*, white in *Beauveria* (Korosi et al., 2019; Maciel-Vergara et al., 2021).

The significance of insect fungi in rearing systems is further emphasized by their natural presence in stored grain, which not only serves as a habitat for *T. molitor* but also as a common source of feed in the insect production facilities (Wakil et al., 2014; Cortes Ortiz et al., 2016). Yeasts have received less attention in insect pathology, including *Hyphopichia burtonii* (Boidin, Pignal, Lehodey, Vey & Abadie) Arx & Van der Walt, a yeast species commonly associated with spoilage of grains

* Corresponding author.
E-mail address: gabriele.bumbulyte@gamtc.lt (G. Bumbulytė).

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and baked goods (Davis, 2015; Bernardi et al., 2019). The yeast has been isolated from a few insect species and interesting that it has been linked to rare cases of peritonitis in humans (Chamroensakchai et al., 2021; Feldman et al., 2023). In addition, its ability to penetrate the skin tissue of living bats (Simpson et al., 2013) raises important questions about its broader pathogenic potential and host range. As mass rearing facilities for insects provide conditions that differ significantly from the natural environment, the dynamics of host-pathogen interactions may shift, potentially allowing for the emergence or increased virulence of opportunistic microorganisms (Slowik et al., 2023).

For several years, the *T. molitor* culture maintained in our laboratory has exhibited signs of disease (e.g., microorganismal growth, sluggish behaviour) that were not previously documented in the literature on *T. molitor* pathogens (Eilenberg et al., 2015; Slowik et al., 2023; Barrett et al., 2024). In this study, we set out to identify the causative agent of the disease and to characterize its effects.

2. Methods

2.1. Insects

Yellow mealworms (*Tenebrio molitor* L.) were fed wheat bran (Malsena plius, UAB, Lithuania) and carrot pieces (MAXIMA LT, UAB, Lithuania) in a box (36 x 26 x 11 cm) at the Laboratory of Chemical and Behavioural Ecology of State Scientific Research Institute Nature Research Centre since 2019. Controlled growth conditions: temperature of 26 °C, 60% relative humidity, and a 12:12 h light–dark (day-light lamps) cycle.

2.2. Isolation of infectious microorganism from t. Molitor

After detection of a yeast colonized beetle, which was cut/opened using a sterile scalpel, a small piece of yeast culture was pinched off with sterile forceps, removed either from the body surface or the internal body tissues and pressed into universal potato dextrose agar (PDA) (Lelliott and Stead, 1987) and malt extract agar (MEA) (Crous et al., 2009) media. The plate containing the material was incubated in an incubator at + 22 °C in the dark until fungal mycelial growth was observed, which took up to 5–10 days. The grown isolate was then transferred to a separate sterile MEA medium and grown at + 25 °C for up to 28 days.

Because we did not perform dissections or histopathological examinations to verify internal colonization of tissues/organs, we use the term *infestation* to describe the observed insect-yeast association rather than *infection*, which would require confirmation of a true internal disease process.

2.3. Identification of the infestation agent

Identification of the microorganism that caused the insect infestation was done through a combination of morphological characterization of the strain cultured on malt extract agar (MEA) medium and molecular analysis. A colony of the microorganism was grown on MEA medium at 22 °C for 7 days. As soon as the fungal mycelium was fully grown, it was examined under the microscope ('Eclipse Ci', Nikon, Japan) and the hyphal structure, spore shape, and size were assessed. The genomic DNA of the strain was obtained using the CTAB method as described by Gardes and Bruns (1993) with slight modifications. A mycelium of cultured fungus was placed in 2 mL tubes with three glass beads (3 mm diameter). 600 µL CTAB solution was added to the tube. The samples were homogenised by shaking twice at 5500 rpm for 30 s, with a pause of 1 min in between. Each time after shaking, the tubes were centrifuged at 9000 rpm for 1 min. The tubes were incubated at 65 °C for up to one hour with occasional shaking. After incubation, 600 µL of chloroform was added to the tubes and shaken well (mixed by hand) and centrifuged at 10,000 rpm for 10 min. The liquid phase was removed (about 500 µL) and poured into 1.5 mL Eppendorf tubes. Two volumes of cold isopropanol were added. The tubes were slowly inverted several times to precipitate the DNA. The tubes were incubated at −20 °C for 60 min or overnight. After incubation, the samples were centrifuged at 13,000 rpm at 4 °C for 30 min. The supernatant was removed, and 200 µL of 70% ethanol was added to gently wash the pellet. The samples were centrifuged for 5 min at 13,000 rpm at 4 °C. The supernatant was removed, and the pellet was dried. 20–50 µL of water was added to each sample to dilute the extracted DNA.

In total, we analysed two nuclear ribosomal regions (ITS and LSU), which were used for the analysis. Primers ITS1 and ITS4 (White et al., 1990) were used to amplify the internal transcribed spacer region, and primers LROR and LR5 (Vilgalys and Hester, 1990) were used to amplify the ribosomal LSU region.

The final volume of the amplification reaction was 15 µL and contained 1.5 µL 10 × PCR buffer (Green Buffer DreamTaq, ThermoFisher, Lithuania), 1.5 µL of dNTPs (2 mM), 0.3 µL of each primer (10 µmol), 0.4 µL $MgCl_2$ (25 mM), 0.075 µL (0.375 U) Taq DNA polymerase, and 1.5 µL fungal DNA (15 ng/µL). The PCR conditions were the same for all analysed loci. PCR was performed in a thermal cycler (Thermocycler Veriti 96 Well Thermal Cycler (Applied Biosystems, USA)). The PCR conditions were a start step of 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at the annealing temperature – 55 °C, 45 s at 72 °C, the final extension step lasted 7 min at 72 °C. The PCR products were visualised in a Tris-acetate agarose gel (1.5%) under UV light. The amplicons were sequenced on both sides (Macrogen, Korea).

The nucleotide sequences were assembled with the software Geneious Prime (version 2025.1.2). The consensus sequences were analysed using a BLASTN search (NCBI GenBank, https://www.ncbi.nlm.nih.gov/). Sequences were then aligned with the representative sequences of type strains of the species using multiple alignments taken from GenBank. A phylogenetic analysis was performed using the maximum likelihood (ML) method of Mega12 software (Kumar et al., 2024). Confidence level of the clades was estimated from bootstrap analysis (1000 replicates). The sequences generated in this study were deposited in GenBank.

2.4. Sampling and analysis of volatile organic compounds (VOCs)

Sampling of volatile organic compounds (VOCs) emitted by healthy or infested T. molitor larvae and adults was performed in vitro by placing purified PDMS/DVB 65 µm Solid Phase Micro Extraction (SPME) fibres (Supelco, USA) in the headspace of an Erlenmeyer flask overnight (approximately 16 h), into which six individuals of either infested larvae, healthy larvae, infested adults, or healthy adults were placed. Three replicates of each group were arranged. The samples were collected at room temperature. Once VOC collection was complete, the SPME fibre was removed from the flask and inserted into a Shimadzu GC-2010 gas chromatograph (GC) coupled with a Shimadzu MS-QP 2010 Plus mass selective detector (MS) for analysis (Shimadzu, Kyoto, Japan). The GC was equipped with a polar DB-Wax column (30 m × 0.25 mm × 0.25 × µm, Agilent Technologies, Santa Clara, CA, USA) and the separation of compounds was performed under the following conditions: the initial temperature of 50 °C was kept constant for 1 min and then increased to 260 °C at a rate of 10 °C/min. The final temperature was maintained isothermally for 17 min. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The electron ionization spectra were recorded at an electron energy of 70 eV; the temperature of the GC injector and the interface were 250 °C, and the temperature of the ion source was 200 °C. MS analyses were performed in scan mode in the range of 33–400 *m/z*. The VOCs were tentatively identified by comparing their mass spectral data and retention indices with the NIST 14 version mass spectra search programme (National Institute of Standards and Technology, Gaithersburg, Maryland, USA).

Sampling of VOCs of *H. burtonii* mycelium isolated from infested larvae and adults was done *in vitro* by exposure of purified PDMS.DVB

65 µm SPME fibers in the headspace of Petri dish. Collection of VOCs was performed for 30 min. and then the SPME fiber was immediately introduced into the GC system (Shimadzu GC-2010), and the profile of collected VOCs was analysed. The analysis conditions were identical to those used to analyse the VOC profiles of *T. molitor* larvae and adults. Sampling and analysis of *H. burtonii* VOCs were done in triplicate.

2.5. Mortality test

The mortality test of *T. molitor* larvae was conducted to compare the impact caused by the yeast *H. burtonii* and the entomopathogenic fungus *Metarhizium anisopliae.* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae). The latter fungus was selected due to its well-established efficacy as an entomopathogen, and the strain used (NRCIB Tm1, GenBank Acc. No. PV716678) was originally isolated from *T. molitor*. The strain was obtained from the collection of the Laboratory of Plant Pathology at the State Scientific Research Institute Nature Research Centre (Vilnius, Lithuania). The test was conducted under controlled conditions: 26 °C, 60% RH, with a 12:12 h Light:Dark cycle.

Three treatment groups were compared: entomopathogenic fungus-infested, yeast-infested, and uninfested larvae of *T. molitor*. A total of 25 larvae per group were used, all at the 1.2–1.4 cm size to ensure developmental consistency. To maximize infestation potential, the entomopathogenic fungus (*M. anisopliae*) was applied at its sporulation stage, when spore viability and infectivity were highest – 7 days old, *H. burtonii* – 2–3 days old. Spores from actively growing fungi were collected using a sterile loop. The spores were transferred to sterile water and counted using a haemocytometer. 200 µl of spore suspension (1×10^7) was transferred to a 0.5 cm piece of agar and gently mixed.

Each group of larvae was fed on an agar-based substrate: one group received agar inoculated with actively sporulating entomopathogenic fungus, another with agar inoculated with the yeast culture, and a control group received sterile agar without microbial inoculation. Larvae were allowed to feed on their respective substrates for three days so that they were exposed to the respective treatment conditions. All that time a piece of agar with spores was left accessible to the larvae. After the exposure period, a small amount of autoclaved wheat bran (about 0.5 g) was added as a food source for the remainder of the test. Survival of larvae was monitored daily for 30 days, with live and dead individuals recorded based on lack of movement and unresponsiveness to tactile stimuli.

2.6. Behavioural test

To assess whether cohabiting uninfested *T. molitor* adults (males and females), can discriminate between larvae infested by *H. burtonii* and uninfested, a two-choice behavioural assay was conducted (Fig. 1).

Adults (10–15 days old) were tested individually in 9 cm diameter plastic Petri dishes configured for dual choice: two 1 cm diameter holes were symmetrically cut in the base, into which tapered–end tubes, wide enough to admit beetles, were inserted. The dishes were covered with lids featuring a central ventilation hole. Each dish contained two 10 mL alternative containers: one with *H. burtonii*–infested one larva, the other with one healthy larva. Male and female adults were tested in separate trials.

Insects were introduced one at a time and maintained under controlled conditions (25 °C, 60% RH, 12:12 L:D) for 24 h. Beetles of each sex underwent 25 replicates. After incubation, the number of beetles that entered each container (infested vs. control) was recorded.

2.7. Statistical analysis

All statistical analyses were performed using R version RStudio 2024.09.1 + 394 (R Core Team, 2023) within the RStudio environment (Posit, 2025). A survival analysis was performed to evaluate the effect of the entomopathogenic fungus on the larvae of *T. molitor*. A Kaplan-Meier survival curve was constructed using the Survival and Survminer packages, and differences in survival distribution between treatment groups were assessed using the log-rank test (Mantel–Cox). The test statistic (χ^2) and *p*-value were calculated to determine statistical significance.

To assess the results of the behavioural assay with infested and healthy *T. molitor* larvae, an exact binomial test was applied to assess preference. Statistical significance was determined at $p < 0.05$, indicating a confidence level of more than 95%.

3. Results

3.1. Case report

In a laboratory colony of *Tenebrio molitor*, infestation was observed in both larvae and adult beetles, with clear external signs of either fungus- or yeast-like colonization.

Initial observations revealed the presence of white, fungus-like spots on various body segments, particularly around the mouthparts, the leg joints, and intersegmental regions, which appeared to be protruding

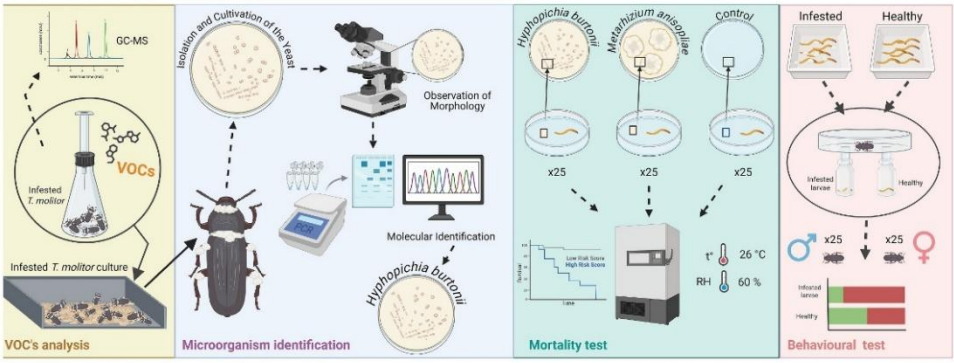

Fig. 1. Principal research scheme. Created in BioRender. Tiškevičiūtė, D. (2025) https://BioRender.com/jc6urv2.

growths of infective microorganisms. The growth appeared as irregularly shaped patches that continued to spread over time.

Movement of the infested individuals was visibly impaired, with the larvae showing sluggish behaviour and reduced responsiveness to tactile stimuli. Some adult beetles showed restricted locomotion, especially those with extensive infestation on the legs and thorax.

The prevalence of visible infectious microorganism colonization increased over time and affected a considerable proportion of the insect colony. No immediate mortality was recorded, but the observed symptoms indicated a progressive impairment of the insect's vitality.

3.2. Identification of the infectious agent

On malt extract agar (MEA), the colonies of the isolates initially appeared smooth, moist, and cream to beige in colour (Fig. 2a). Over time, they became opaque and developed a wrinkled or folded surface with a leathery to slightly powdery texture. The edges of the colonies often became irregular. Microscopically, the isolates exhibited both yeast-like and filamentous growth, characterised by the formation of asci from conjugating yeast cells and the development of septate hyphae and conidia borne on denticles. The cells had an oval to elongated shape and formed septate, branched hyphae (Fig. 2b).

To identify the species of the microorganism that was found in adult beetles and larvae, molecular analyses were conducted. Sequencing the internal transcribed spacer (ITS; GenBank Acc. No. PV716683) and the large subunit (LSU; GenBank Acc. No. PV716685) regions were performed, and the results were compared with reference sequences from representative strains. The LSU sequence showed 100% identity with the CBS 2352 strain (GenBank Acc. No. NG_054819), while the ITS sequence showed 99.10% identity with the same strain (GenBank Acc. No. KY103604).

Based on both molecular and morphological characteristics, the isolate was identified as *Hyphopichia burtonii* (strain registered as NRCIB Hp1). The morphological features of the isolate were consistent with those of descriptions of *Hyphopichia burtonii*, as reported by Groenewald and Smith (2010) and Kurtzman (2011).

3.3. Volatile organic compounds (VOCs)

The analysis of VOCs revealed distinct chemical profiles among *T. molitor*, either infested or healthy, both larvae and adults, as well as isolated *H. burtonii* yeast (Table 1).

Infested larvae showed higher diversity of emitted chemical compounds compared to healthy ones: 23 compounds exceeding the concentration of trace level were registered, while 16 VOCs were registered in healthy ones. The prevailing components were clearly different as well: hexanoic acid (up to 7.35%) and two esters (propanoates: 1-[2-(isobutyryloxy)-1-methylethyl]-2,2-dimethylpropyl 2-methylpropanoate and 1-(2-hydroxy-1-methyle)-2,2-dimethylpropyl 2-methylpropanoate) (up to 5.11% and up to 4.29% correspondingly) were the main compounds released by healthy larvae, while two terpenes, namely limonene (up to 10.79%), *trans*-caryophyllene (up to 4.66%) and nitrogen, containing compound 2,4,6-trimethyl-pyridine (up to 3.52%) prevailed in the emissions of *H. burtoni* infested *T. molitor* larvae. The VOCs of the infested larvae contained the most notable reduced level (approximately twice) of aldehyde nonanal and heptanoic acid (nearly three times) compared to those of healthy larvae (Table 1).

In adults, the VOC profile of infested beetles compared to healthy insects was slightly richer in the number of detectable (exceeding trace level) compounds (13 versus 10). The prevailing components were clearly different. In healthy adults, the most abundant were: *cis*-3-dodecen-1-ol acetate (up to 12.01%), followed by 2,5-dimethyl-pyrazine (up to 9.19%) and polycyclic hydrocarbon (2,3,4,5,6,7-hexahydro-3a,6-methano-3aH-indene) (up to 6.04%). In infested prevailed limonene (up to 4.07%), followed by 2,5-dimethyl-pyrazine (up to 3.5%) and *cis*-5-dodecen-1-ol acetate (up to 1.46%), alongside the above-mentioned polycyclic hydrocarbon (up to 1.53%). When analysing groups of the compounds, pronounced differences in the concentrations of separate components within the group of ketones were not recorded. In other VOC groups, some compounds regarding the concentration were modified more distinctly. Among alcohols relative amount of *cis*-11-tetradecen-1-ol, among aldehydes, that of nonanal, among acids, that of hexanoic acid, and among esters, that of *cis*-3-dodecen-1-ol acetate and tridecyl acetate were reduced; however, within the group of terpenes concentration of a single compound, namely limonene, increased dramatically comparing infested/healthy beetles.

The VOC profile of yeast *H. burtonii* was distinctly different from all those of both infested and uninfested insect larvae and adults (Table 1). Diversity of chemical compounds released by H. *burtonii* yeast was poor, and only 9 compounds were recorded at concentrations exceeding trace level. The prevailing group of alcohols with distinctly predominated by 3-methyl butanol (up to 64.13%), followed by ethanol (up to 9.69%) and phenylethyl alcohol (up to 6.89%) (Table 1). Neither of the three alcohols nor isobutyl alcohol, characteristic of *H. burtonii* volatiles, were detected in infested or healthy insects, neither in larval nor adult stages.

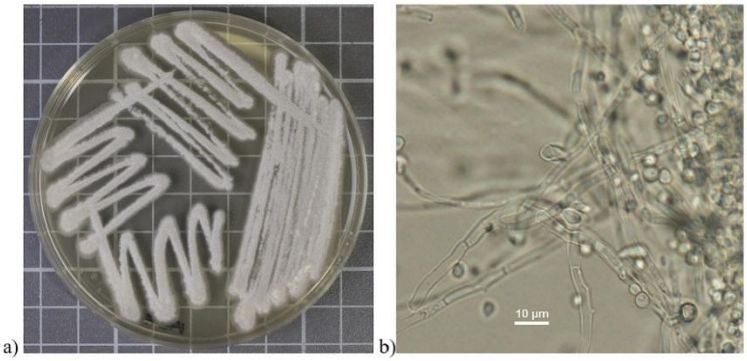

Fig. 2. *Hyphopichia burtonii* strain NRCIB Hb1 colonies on malt extract agar on day 7 (a), hyphae and conidia (b).

Table 1
Relative amounts of VOCs emitted by healthy and infested larvae and adults of *Tenebrio molitor* and a pure culture of *Hyphopichia burtonii* yeast.

Compound	Amount interval (min-max), %				
	Larvae infested	Larvae healthy	Adults infested	Adults healthy	*H. Burtonii*
Alcohols					
1-Hexanol	0.1—0.2	tr – 0.43	nd – 0.1	nd - 0.30	nd
2-Ethyl-1-hexanol	1.86—2.13	1.72—1.95	1.00 – 1.31	1.07 – 1.71	nd
Ethanol	nd	nd	nd	nd	5.03—9.69
Isobutyl alcohol	nd	nd	nd	nd	3.94—4.65
3-Methyl butanol	nd – tr	nd	nd – tr	nd	59.13—64.13
Phenylethyl alcohol	nd	nd	nd	nd	6.02—6.89
cis 11 Tetradecen 1 ol	nd	nd	0.2—0.57	0.12—1.35	nd
Aldehydes					
Nonanal	nd – 1.69	0.5—3.15	nd – 0.8	1.89—2.77	nd
Ketones					
6-Methyl-5-hepten-2-one	0.13—0.32	0.27—0.54	nd – 0.1	nd – 0.75	nd
6,10-Dimethyl-(*E*)-5,9-undecadien-2-one	0.44—0.56	0.31—0.9	0.38—0.69	0.34—1.21	nd
Acetophenone	nd	nd	nd	nd	0.1—0.14
Terpenes					
α Pinene	0.84—1.5	nd	0.11—0.51	nd	nd
β-Pinene	tr – 0.1	nd	tr – 0.18	nd	nd
Sabinene	tr – 1.62	nd	nd	nd	nd
Myrcene	0.56—0.78	nd	nd – tr	nd	nd
Limonene	9.67—10.79	nd	6.99—8.13	nd	tr – 0.16
Caryophyllene	0.62—4.66	nd – 0.12	nd	nd	nd
Acids					
Hexanoic acid	0.59—1.97	2.05—7.35	0.45—4.15	1.15—6.05	nd
Heptanoic acid	nd – 0.99	1.37—3.00	nd – 0.94	1.82—4.54	nd
Octanoic acid	tr – 0.35	0.1—0.86	nd – 0.29	tr – 0.51	nd
Nonanoic acid	tr – 0.46	tr – 0.63	0.06—1.13	0.34—2.98	nd
3-Methylbutanoic acid	nd	nd	nd	nd	0.34 – 0.66
2-Methylbutanoic acid	nd	nd	nd	nd	0.26 – 0.49
Esters					
3-Methyl-butanoic acid propyl ester	tr – 0.58	nd – tr	nd	nd	nd
3-Methylbutyl 3-methylbutyrate	0.89—2.02	1.99—2.29	nd	nd	nd
cis 2 Hexen 1 ol acetate	0.1—0.37	nd – tr	nd	nd	nd
Tridecyl acetate	nd	nd	tr – 0.07	0.45—1.28	nd
2-Ethyl 3-hydroxyhexyl 2 methylpropanoate	2.0—2.45	2.17—4.05	0.4—1.04	nd – tr	nd
1-[2-(Isobutyryloxy)-1-methylethyl]-2,2-dimethylpropyl 2-methylpropanoate	0.66—3.6	3.03—5.11	0.66—1.24	3.04—4.75	nd
1-(2-Hydroxy-1-methyle)-2,2dimethylpropyl 2-methylpropanoate	0.1—1.83	2.95—4.29	0.63—1.06	0.61—4.15	nd
3-Methyl-1-butyl acetate	nd	nd	nd	nd	0.82—1.4
cis-5-Dodecen-1-ol acetate	nd	nd	1.28—2.91	nd	nd
cis-3-Dodecen-1-ol acetate	nd	nd	nd	7.39—12.01	nd
Nitrogen compounds					
Trimethylamine	nd	tr – 2.27	tr – 0.07	nd	nd
Triethylamine	0.27—1.38	nd – tr	nd	nd	nd
2,5-Dimethyl-pyrazine	nd	nd – 0.29		9.19—10.74	nd
2,4,6-Trimethyl-pyridine	3.52	tr – 0.17	tr – 0.34	nd – tr	nd
Aromatic hydrocarbons					
Toluene	tr – 0.38	nd –0.13	nd –tr	nd	nd – tr
para-Xylene	0.32—1.06	0.15—1.02	tr – 0.19	tr – 0.16	0.9—1.36
Polycyclic hydrocarbons					
2,3,4,5,6,7 Hexahydro 3a,6 methano 3aH indene	nd	nd	1.85—3.05	2.83—6.04	nd
Total No of compounds exceeding tr level	23	16	13	10	9

nd – not detected; tr – traces ($\leq$ 0.01%).

3.4. Comparative mortality test

Preliminary observations of the behaviour of *H. burtonii*-infested larvae indicated that their locomotory activity was suppressed. To assess negative effect, we compared the yeast-infestation with that of entomopathogenic fungus. For comparison *Metarhizium anisopliae* was chosen. The survival curves of *T. molitor* larvae exposed either to entomopathogenic fungus *M. anisopliae*, or yeast *H. burtonii*, or remained intact (control) (Fig. 3) showed a clear separation, with no mortality observed in the control and yeast-infested groups during the seven-day testing period. In contrast, larvae exposed to the entomopathogenic fungus exhibited progressive mortality, with the first deaths occurring on day 3, followed by a steep increase.

Survival differed significantly between the three groups (log-rank test, $\chi^2 = 93.1$, df = 2, $p < 0.0001$). During the test period, no deaths were recorded in the control and yeast affected groups, indicating no negative effect. In contrast, all 25 larvae in the group with the fungal treatment died by day 7, which emphasises the strong pathogenic effect of the entomopathogenic fungus *M. anisopliae* on *T. molitor* larvae.

The data obtained demonstrated that mortality impact of the yeast *H. burtonii* on *T. molitor* larvae cannot be matched to that caused by entomopathogenic fungus *M. anisopliae*.

3.5. Behavioural test

To reveal if the spreading of *H. burtonii* within the culture of *T. molitor* can be limited via adults' avoidance of infested larvae, whose smell has changed compared to healthy ones, a two-choice behavioural test was carried out. Preliminary test of the device used revealed equal distribution of *T. molitor* adults with no statistically significant difference in the choice of any of the two-alternative pit-falls.

Adult beetles showed no significant preference between uninfested and infested larvae ($p > 0,05$), with only slight, statistically non-significant trends observed in both male and female choice behaviour (Fig. 4).

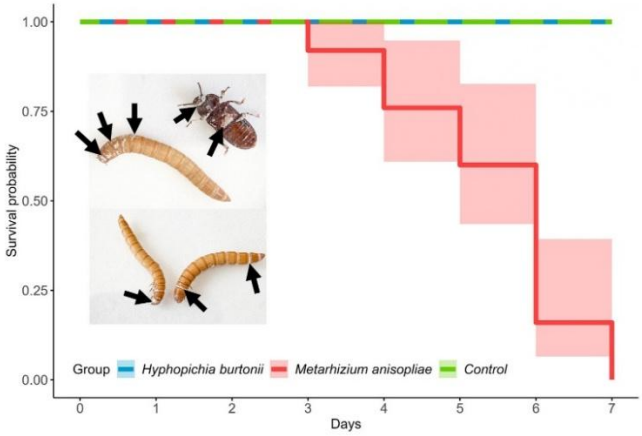

Fig. 3. Kaplan-Meier survival curve of *Tenebrio molitor* larvae exposed either to entomopathogenic fungi *Metarhizium anisopliae* or yeast *Hyphopichia burtonii* and intact (control). Inner graph: *T. molitor* larvae and adult beetle infested (arrows indicate) by *H. burtonii*.

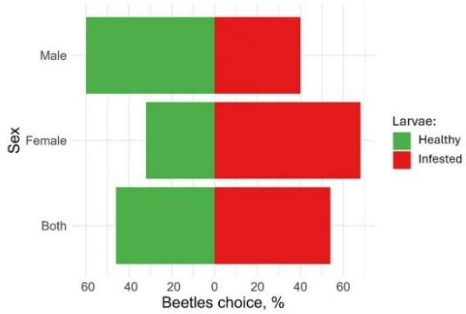

Fig. 4. Choice of *Tenebrio molitor* adult's female and male between infested *Hyphopichia burtonii* larvae and healthy.

4. Discussion

The identification of *Hyphopichia burtonii* in both larvae and adults of *Tenebrio molitor* reveals a previously underexplored aspect of microbial dynamics in insect rearing systems. While *H. burtonii* is typically associated with spoilage in stored grains and bakery products (Davis, 2015; Bernardi et al., 2019), its presence in insects suggests that artificial rearing environments may facilitate colonization by opportunistic microorganisms. This aligns with observations that high-density rearing conditions can alter both insect physiology and microbial behaviour, potentially enabling benign microbes to acquire pathogenic traits (Slowik et al., 2023).

Insect-yeast interactions represent a complex and increasingly recognized component of insect ecology, encompassing relationships that range from mutualistic symbiosis to neutral associations and opportunistic antagonism. Yeasts are commonly associated with insects through the gut or dietary substrates, where they may contribute to host nutrition by supplying limiting nutrients, enzymes, and sometimes by modulating behaviour via VOCs that influence either foraging or oviposition decisions (Malassigné et al., 2021; Mozūraitis et al., 2022; Biasazin et al., 2022; Holt et al., 2024). However, many of these associations are facultative, with insect mycobiota composition strongly shaped by diet and environmental conditions (Malassigné et al., 2021). Compared with filamentous entomopathogens, yeast associated insect diseases are reported less frequently.

The yeast *H. burtonii* is mentioned in studies of insect associated yeasts. In the coffee berry borer (*Hypothenemus hampei*), *H. burtonii* was recovered from feces and surface-sterilized, whole-insect homogenates, which demonstrates presence in internal tissues (Pérez et al., 2003; Vega et al., 2003). Also, the yeast recovered from pollen carried by wild bees and yeast-insect associations were documented without revealing any function (Kurtzman, 2011). *H. burtonii* was isolated from the surface of stored product coleopteran pest, weevil *Sitophilus zeamais* (Coleoptera: Curculionidae), and it was established that the yeast suppressed key mycotoxigenic fungi, suggesting community level benefits for the insect which lives in grain and feed on them. Therefore, the yeast could indirectly influence host fitness (Seco et al., 2024).

In the present paper, to our knowledge, *H. burtonii* was identified in *T. molitor* insects for the first time. This yeast was also recorded for the first time as an agent causing disease in insects, both larvae and adults.

The frequent use of grain-based substrates in *T. molitor* rearing (Bumbulytė et al., 2023; Vrontaki et al., 2024) likely increases exposure to grain-associated yeasts such as *H. burtonii*. Notably, this yeast has demonstrated tissue penetration in other hosts (Simpson et al., 2013) and has been implicated in rare human infestation s (Chamroensakchai et al., 2021; Feldman et al., 2023), raising questions about its pathogenic potential in insects.

One of the most striking findings was the alteration of volatile organic compound (VOC) profiles in infested insects. Infested larvae and adults emitted more VOCs than their healthy counterparts, with significant shifts in compound composition. For example, healthy larvae predominantly released hexanoic acid and propanoate esters, while infested larvae exhibited elevated levels of limonene, *trans*-caryophyllene, and 2,4,6-trimethyl-pyridine. These terpenes were absent in healthy individuals, suggesting that their presence is linked to yeast infestation rather than diet or environmental factors. Limonene is known for its broad bioactivity, including antimicrobial and insecticidal properties (Lin et al., 2024), and can be synthesized by yeasts such as

Saccharomyces cerevisiae under specific conditions (Kong et al., 2023). The detection of *trans*-caryophyllene exclusively in infested larvae further supports the hypothesis that *H. burtonii* modulates its metabolism in response to host-derived substrates.

In adults, similar but less pronounced VOC changes were observed. The reduction of *cis*-3-dodecen-1-ol acetate – a known sex pheromone in *T. molitor* (Bryning et al., 2005) – suggests potential disruption of reproductive signaling. Despite these changes, behavioural assays indicated that beetles do not distinguish between infested and healthy conspecifics based on scent, implying that the infestation may spread undetected within populations. This is particularly relevant given that limonene, the most abundant VOC in infested individuals, does not appear to be perceived by the insects.

Our research demonstrated that *H. burtoni* releases a considerable amount of limonene when interacting with an insect. This volatile VOC possesses antimicrobial properties (Lin et al., 2024) and may therefore be responsible for the suppression of mycotoxigenic fungi observed by Seco et al. (2024). However, limonene also has insecticidal properties (Lin et al., 2024), so the assumption of a positive influence of VOCs on beetle fitness (Seco et al., 2024) might not be accurate and should be studied further.

VOC analysis of *H. burtonii* in pure culture revealed a profile dominated by alcohols, such as 3-methyl butanol and phenylethyl alcohol, with only trace amounts of limonene. This contrasts sharply with the VOCs emitted by infested insects, indicating that host tissue significantly influences yeast metabolism. Similar discrepancies were reported by Pina et al. (2009), who found limonene only in trace amounts when *H. burtonii* was cultured on standard media. These findings suggest that the host environment may activate biosynthetic pathways not typically expressed *in vitro* (Xiao et al., 2016; Davis et al., 2014).

Importantly, no VOCs hazardous to humans were detected in either infested insects or yeast cultures. Although *H. burtonii* did not cause mortality within the timeframe typical for entomopathogenic fungi (e.g., ~7 days for *M. anisopliae*), its ability to alter host biochemistry and potentially interfere with reproductive signaling warrants further investigation. The consistent increase in limonene across developmental stages may serve as a biochemical marker of infestation, though its utility for early detection remains to be validated.

5. Conclusions

This study shows that *Hyphopichia burtonii* can colonize *Tenebrio molitor* and significantly change its VOC emissions, especially increasing limonene levels. These changes indicate a complex interaction between the host and microorganism, where the yeast adjusts its metabolism to the host environment. Given that *H. burtonii* infestation is observed in laboratory colonies, it may nonetheless be associated with changes in insect behaviour, reproduction, and the microbial ecology of rearing systems. Limonene emerges as a potential marker of infestation and deserves further study for early detection and management in insect farming. Future work should include dissections and histopathological (and, where appropriate, culture- or molecular-based) assessments to determine whether the beetles exhibit true internal infection or whether this represents only external/surface-level infestation.

CRediT authorship contribution statement

Gabrielė Bumbulytė: Writing – original draft, Methodology, Investigation, Formal analysis. **Dovilė Čepukoit:** Methodology, Investigation, Formal analysis. **Jurga Būdienė:** Investigation, Formal analysis. **Daiva Burokienė:** Methodology. **Vincas Būda:** Writing – review & editing, Conceptualization.

Declaration of competing interest

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

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PAPER V

Wheat grain infected by *Aspergillus flavus* Link modifies behavior of yellow mealworm, *Tenebrio molitor* L.

Bumbulytė, G., Lučinskaitė, I., Čepukoit, D., Būda, V.

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Search for *Tenebrio molitor* behaviorally active compounds in *Aspergillus flavus*-infected grain

Gabrielė Bumbulytė[1*], Ieva Lučinskaitė[1], Dovilė Čepukoit[2], Vincas Būda[1]

[1]Laboratory of Chemical and Behaviour Ecology, Nature Research Centre, Akademijos Str. 2, LT-08412 Vilnius, Lithuania

[2]Laboratory of Plant Pathology, Nature Research Centre, Akademijos Str. 2, LT-08412 Vilnius, Lithuania

*Corresponding author gabriele.bumbulyte@gamtc.lt (G.Bumbulytė)

Abstract

Application of insecticides for pest control must be minimized due to well-known risks to human health. Application of environmentally friendly means is the best option. For stored product pests' control, new means are needed alongside a deep understanding of the ecology and behaviour of the pests. The present study investigated whether the odor released from wheat grain infected by mold fungus *Aspergillus flavus* influences behaviour of the yellow mealworm (*Tenebrio molitor* L.), a stored product pest of economic importance with worldwide distribution. Analysis of video-recorded behaviour and computer data analysis revealed a significant difference in reactions depending on the beetle's sex. Females were more sensitive to fungus contamination as they discriminated between *A. flavus*-infected and uninfected wheat grains from the onset of infection, while males discriminated only after five days period. Analysis of single VOCs revealed the presence of both repellents and attractants. Among those, hexanal was repellent for both males and females, 1-octen-3-ol was repellent for males and indifferent for females, 1-hexanol was attractive for females and repellent (at high concentrations only) for males; 3-methylbutan-1-ol was attractive to females (at high concentrations only) and indifferent for males. Hexanal, as a potential repellent, was suggested for further testing under grain storage conditions.

Introduction

The application of insecticides for pest control must be significantly reduced due to their risks to human health. The use of ecologically safe approaches is the best option. Among the alternatives to insecticides as environmentally friendly control methods for stored product pests (including *Tenebrio molitor* L. (Coleoptera, Tenebrionidae)), application of entomopathogenic nematodes, genetic techniques, and some others are being proposed (El-Husseini, 2006; Koc et al., 2022; Prabowo et al., 2019; Alphey and Bonsall, 2018). Research on the evaluation of the effectiveness of plant essential oils (EOs) is going on (Campolo et al., 2018; Kavallieratos et al., 2022; Bumbulytė et al., 2023; Danna et al., 2024). Control of stored product pests through the application of natural repellents appears promising as well (Stankovic et al., 2020).

EOs are composed of a complex mixture of volatile organic compounds (VOCs), among which only specific constituents are responsible for the observed biological activity. For example, in EOs from several plant species that repel adult *T. molitor*, only 2-3 active components have been identified (Bumbulytė et al., 2023). The use of such active components would significantly reduce the costs associated with the application of complex EO. Searching for synthetic analogs of biologically active EOs' components is a promising, though remains underexplored, field.

Although numerous laboratory studies have examined EOs and VOCs that affect *T. molitor* behavior, the chemical space of candidate actives should be broadened beyond EOs to include microbial (fungal) volatiles. To that, we believe that a possible source of environmentally friendly VOCs could be *T. molitor* substrate infected with fungus.

Since the application of repellents requires as effective chemical compounds as possible, it makes sense to expand their list by searching for extra compounds. Another possible natural source of them could be VOCs emitted by mold fungi, infecting stored grain, i.e. compound from volatiles released by competitor for food with stored product pests. It is known that moldy grain

53 negatively affects the development of *T. molitor* larvae and can even be toxic to them (Reiss,
54 1973). Therefore, through co-evolution, insects may have developed mechanisms to recognize
55 such spoiled substrates. If recognition occurred only after the insect's contact with the fungus, the
56 insect could become a potential spore carrier and spread the fungus, which would be highly
57 disadvantageous for the insect. The ability to recognize fungus-infected grain from a distance
58 would be beneficial for the insect. Such recognition could be facilitated by VOCs released into the
59 environment. It is known that some other storage pests, such as the moth *Plodia interpunctella*
60 Hübner (Lepidoptera, Pyralidae), can recognize substrates infected by various species of mold
61 fungi, and sheared VOC involved in this process have been identified (Būda et al., 2016).
62 The aim of the present study was to check if natural emissions of VOCs released by wheat
63 grain infected with the fungus *Aspergillus flavus* Link (Eurotiales, Aspergillaceae) were repellent
64 for *T. molitor* adult beetles, and, if such an effect exists, to reveal behavioural impact of the main
65 components. Since the VOC composition of the fungus *A. flavus* has been thoroughly studied
66 (Kaminśkiet al., 1976; Börjessonet al., 1989; Magan, Evans, 2000; Duan et al., 2021; Kang et al.,
67 2022; Lin et al., 2023; Chen et al., 2024), the compounds for the research were selected based on
68 already known data.

69 ## Materials and methods

70 ### Insects

71 *Tenebrio molitor* culture was maintained at the Laboratory of Chemical and Behavioural
72 Ecology, Nature Research Centre, Vilnius, Lithuania, since 2019. The insects were reared on a
73 diet consisting of a mixture of wheat bran (90%), plus dry yeast (10%), and provided with 10 g of
74 carrot slices. During rearing, temperature of 25 °C, relative humidity of 60%, and a 12:12 h light-
75 dark cycle was maintained. Beetles aged 9-10 days were used for the study. The sexes of the pupae

were distinguished based on distinct features: female pupae have a pair of prominent papillae on the last abdominal segment.

Fungus

Aspergillus flavus culture was obtained and cultivated at Laboratory of Biodeterioration Research, Nature Research Centre, Vilnius. The fungal culture was cultivated on potato dextrose agar (PDA) (Lelliot and Stead, 1987) at 25 °C for 7 days (or until spores were formed).

Composition of potato dextrose agar: D-glucose 10 g/L (Roth, Germany), agar (microbiological) 20 g/L (Roth, Germany), potato extract 200 ml/L, distilled water.

The prepared media was autoclaved at 125 °C (Autoclav MultiControl, CertoClav, Austria) at a pressure of 1,2 atm and then dispensed into Petri plates (90 mm) following a cooling period.

The fungi were cultivated at a temperature of 25 °C. To prevent cross-contamination, vials (7 ml) containing the grains (2 g) were autoclaved at 125 °C at 1.2 atm. The grains were inoculated with the pathogenic fungus *A. flavus*. The spores were collected with a sterile loop from a Petri plate, where each one was fully colonised (spores formed) at the age of 7 days. Subsequently, the spores were transferred to a vial containing 2 g of grain and 300 µl of water, where they were evenly distributed throughout the grain (Figure 2.2). Grain has been wetted with sterile water to maintain moisture. The fungi with the grains were incubated for periods of 0, 5, 10 and 20 days.

All procedures were conducted within a sterile laminar flow cabinet (NUAIRE; Class II, USA). The samples were then incubated in a thermostat at 27 °C for an appropriate duration (Thermostat Binder BD 115 (WR/Binder, Germany)).

Chemical compounds

Analytical standards used in the tests were 1-octen-3-ol (analytical standard, ≥98% ,GC), hexanal (analytical standard, ≥98%, GC), 3-methylbutanol (analytical standard, ≥99%, GC), 1-pentanol (analytical standard, ≥99%, GC), toluene (ACS reagent, ≥99.5%) (Sigma-Aldrich

(Darmstadt,Germany)), 1-hexanol (analytical standard, ≥99%, GC) (Fluka (Charlotte, NC, USA)), and 2-methylfuran (≥98%) (Toronto Research Chemicals Inc. (Toronto, ON, Canada)).

Behavioural test

For evaluating the effect caused by *A. flavus*, a pit-fall test was used. The experiment was carried out in a plastic Petri plate with a diameter of 9 cm in a two-choice test. Holes of 1 cm were cut in the plate at equal distances from the edge. Into these holes, 1 cm diameter tubes were inserted, with the tapered ends that allowed the beetles to pass through. The plates were covered with a Petri plate, which contained small holes made in the middle for air circulation. Two 10 mL bottles were used per Petri plate, one with wheat grain infected by *A. flavus* (AF) and another one with wheat grain only (control – C). In the control, without stimulus, only wheat with water was used (Figure 1A). Males and females were tested separately.

The beetles were placed in Petri plates for testing one by one and kept for 24 h at 25 °C, 60 % humidity, with a 12:12 light/dark period. Fifteen replicates were performed for each condition, with fungal incubation times of 0, 5, 10, and 20 days.

After 24 h, the number of beetles that selected the control and, respectively, those that selected the fungus-infested wheat grain days were counted.

For evaluation effect caused by single volatiles, behavioural test was performed in a Petri plate 10.5 cm in diameter and supplied with filter paper on the bottom. To one side of the plate, a stimulus consisting of a single chemical compound in solvent was applied 2-3 mm from the edge. On the opposite side of the plate, a solvent (control) was applied at the same distance from the edge as the stimulus. The chemical compound was applied in five consecutive spots (Figure 1B), each containing 2 µL, for a total of 10 µL. The solvent was applied in the same manner and volume. For testing the effects caused by single compounds, three concentrations of each were used: 1 mM, 10 mM, and 100 mM. The doses were 0.01 mM/mL, 0.1 mM/mL, and 1 mM/mL. Hexane was used as a solvent and used in control. After presenting the stimulus and control for approximately

0.5 min to allow the solvent to evaporate, a single *T. molitor* adult was placed at the center of the plate and allowed to move freely for 5 min; the time spent in the stimulus and control zones was recorded. The plate was positioned under a daylight lamp, and air exhaust was adjusted to prevent the accumulation of volatiles. The test was conducted at a temperature of 23 ± 2 °C.

The adult behaviour was recorded for five minutes using a video camera, and the footage was analyzed with EthoVision XT v. 12, computer-based animal behaviour analysis software developed by Noldus (the Netherlands). Ten recordings were analyzed for each stimulus tested, and each adult beetle was used only once. The analysis of the *Tenebrio* behaviour in both the control and stimulus zones was based on time spent in each zone.

Statistical Analysis

Statistical analysis was conducted using Microsoft Excel (version 2501), R version 4.3.1 (R Core Team, 2023) within the RStudio environment (RStudio Team, 2023), and STATISTICA software (StatSoft Inc., version 12). For the two-choice pitfall test with wheat grain infected by *Aspergillus flavus*, the experimental unit was a single beetle, and preference was defined as the choice of infected grain (success) versus uninfected grain (failure). For each sex and fungal incubation period (0, 5, 10, and 20 days), 15 independent replicates were performed; a two-sided exact binomial test was used to test whether the proportion choosing infected grain differed from 0.5. For the two-choice assay with individual VOCs (Petri dish), the experimental unit was a single beetle, and time spent (s) was recorded for both the stimulus and control zones, yielding paired observations. For each compound and concentration, 10 independent beetles were tested (each beetle used once). Differences in time spent between the stimulus and control zones were analysed using a two-sided Wilcoxon signed-rank test (paired design). A non-parametric test was used because time data were bounded (0-300 s) and did not meet normality assumptions. Statistical significance was set at $p<0,05$.

VOC composition

Since there have already been publications on the VOCs of grain infected by fungi, literature search was conducted in Google Scholar, Web of Science, PubMed, and Scopus, using a wide range of keywords including *A. flavus,* wheat, and VOCs. The analyzed papers were limited to those in which the microscopic fungus was cultivated exclusively on wheat culture, and not on other substrates.

To test the impact of active compounds in *A. flavus* on the behaviour of adult *T. molitor* beetles, seven compounds were chosen (Table 1) based on their recurrence in at least three publications.

Results

Recording the choice behaviour of *T. molitor* adults under a control (preliminary) test, with no chemical stimulus in any of the alternative vials, revealed equal distribution with no statistically significant difference in the choice of any of the two alternative vials.

Infected/uninfected wheat grain

The choice between uninfected and *A. flavus* infected wheat grain differed from the choice in the control assay. Statistically significant difference was recorded for both females and males. The beetles preferred uninfected grain to that infected by *A. flavus (*Figure 3). However, sex-specific differences were revealed.

Adult females avoided fungus-infected wheat grain since the very beginning of infestation (following fungus application, "zero day"). Males started avoiding later, starting day 5 ($p<0.05$) and maintained the choice onwards (Figure 3).

Single compounds' effect

For a more detailed study of behavioural reactions to single compounds, an automatic behavioural recording system was used and tested with no chemical stimuli application before the assay. In the

absence of chemical stimulation, most of the time, beetles are spent in the peripheral zone along the edges of the arena. Therefore, for further detailed testing of chemical compounds' effect, insect behaviour in the peripheral part of the arena was recorded and analyzed, namely in a 1.5 cm wide track along the arena's wall, referred to further as a zone. Before testing, the time spent by *T. molitor* adults in the left and right zones of the arena was assessed. No significant difference (Wilcoxon matched pairs test, $p>0.05$) was recorded.

When single compounds from wheat grain infested *by A. flavus* VOCs were presented, the mealworm adults modified their behaviour.

1-Octen-3-ol

The behavioural reaction of *T. molitor* males and females to this compound differed. The compound was repellent for males, and the effect was statistically significant starting from the lowest concentration) tested (1 mM, Figure 4A), ($p<0.05$). However, the compound was neither repellent nor attractive for females, since the duration spent on the stimulus and control zones did not differ when any concentration of 1-octen-3-ol (1; 10; 100 mM) was tested ($p>0.05$) (Figure 4B).

Hexanal

In adult males, 1 mM concentration of hexanal caused no statistically significant repellent effect, even though more time was spent in the control zone than in the stimulus one ($p>0.05$). At higher dosages, 10 mM and 100 mM, hexanal was repellent, as the time spent in the control zone was statistically significantly higher compared to that in the stimulus zone ($p<0.05$) (Figure 5A). For adult females, all concentrations of hexanal tested (1, 10, and 100 mM) were repellent, as a statistically significant difference between time spent in the control and the stimulus zone was recorded ($p<0.05$) (Figure 5B).

1-Hexanol

198 The chemical compound was repellent for males just at the highest concentration tested
199 (100 mM), since the time spent by males in the control zone was significantly higher than in the
200 stimulus zone ($p<0.05$) (Figure 6A). The effect on females was the opposite. Adult females were
201 attracted to 1-hexanol from 1 mM to 10 mM concentration, as the time spent in the control zone
202 was significantly higher than in the stimulus zone ($p<0.05$). This attractive effect ceased at 100
203 mM concentration (no statistically significant difference in time spent within the control and
204 stimulus zones was recorded ($p>0.05$) (Figure 6B).

205 **3-Methylbutanol**

206 The chemical compound was found to have no repellent effect for males in all
207 concentrations tested; the time spent in the control zone was not significantly higher than in the
208 stimulus zone ($p<0.05$) (Figure 7A). In adult females, 3-methylbutanol was attractive just when a
209 high concentration of the compound was presented (1 mM) only, as a statistically significant
210 difference in time spent within the control and stimulus zones was recorded ($p>0.05$) (Figure 7B).

211 **1-Pentanol, 2-methylfuran, toluene**

212 The time spent by the adults (both males and females) in both the control and stimulus
213 zones was similar, and no significant differences across all tested concentrations (1, 10, and 100
214 mM) ($p>0.05$) were recorded.

215 In general, the study on behavioural reactions of *T. molitor* adults to VOCs released by
216 wheat grain contaminated with fungus *A. flavus* revealed repellent properties of such a substrate.
217 Among the VOCs released by the fungus, compounds with both repellent and attractive properties
218 (just for females) were identified (Table2). The effect depended both on the concentration of the
219 compound and the sex of the insect. The following VOCs were found to repel *T. molitor* adults: 1-
220 octene-3-ol was repellent to males of *T. molitor* only from the lowest concentration studied (1
221 mM). Hexanal was repellent to males from 10 mM and to females from 1 mM. 1-Hexanol showed
222 repellent effects in males at a concentration of 1 mM and was attractive to females (at all

concentrations tested). 3-Methylbutanol was found to attract females at a concentration of 100 mM. Three compounds (1-pentanol, toluene, and 2-methylfuran) evoked no behavioural reactions in the studied beetles. In a very generalized form, the results are presented in Table 2.

Discussion

The results of this study demonstrated that *T. molitor,* both females and males, distinguish fungi-infected *versus* non-infected wheat grain from a distance, i.e., by sense of olfaction, with different sensitivity: females were capable of detecting infected grain on the same day as the infection, meanwhile males later, starting from the fifth day of the infection. Therefore, it is obvious that females' behavioural reaction to VOCs emitted by *A. flavus* infected grain suggests that their olfactory sensitivity is much higher compared to that of males. This sex-specific behaviour underscores the complexity of insect-fungus interactions and raises questions regarding the underlying mechanisms driving these responses. In our opinion, among the driving forces may be higher selectivity of females to the quality of substrate used for egg laying, as their reproductive success depends on the right choice of substrate; therefore, they are more likely to avoid an environment that could threaten offspring.

The effects of the seven most characteristic compounds that are released from *A. flavus* - infected wheat grain were analyzed in detail.

1-Octen-3-ol, commonly produced VOC by many microscopic fungi such as *Fusarium* sp., *Penicillium* sp., *Aspergillus* sp., and *Alternaria* sp. (Jele´n & Wasowicz, 1998; Combet et al., 2006), has been demonstrated to exhibit significant insecticidal properties across different developmental stages of the red flour beetle (*Tribolium castaneum* Herbst (Coleoptera, Tenebrionidae) (Cui et al., 2021). In a behavioural test, 1-octen-3-ol was attractive to rice weevil *Sitophilus oryzae*, which is not surprising, because the source of the main food of this beetle (red brown rice) releases this compound in abundance (Cao et al., 2024). Our tests revealed that 1-

247 octen-3-ol has repellent properties and repels adult *T. molitor* beetles. However, repellent property
248 was species-dependent: the compound repelled yellow mealworm adult males, but not females
249 (under the same range of concentrations). Since males are sensitive to this compound, not females,
250 1-octen-3-ol is not the compound that determines the effect of the whole bouquet of VOCs of *A.*
251 *flavus*-infected wheat grain on females (discussed above).

252 In Tenebrionids, there was no data on behavioural or EAG response to hexanal. Our data
253 revealed the repellent effect of the compound on *T. molitor* adults (both females and males). Since
254 males were less susceptible to this compound than females, it was considered to be the main
255 component that determines the repellent property to *T. molitor* beetles by fungus infected grain. In
256 our test, hexanal turned out to be the most effective repellent for male and female yellow
257 mealworms. It is necessary to note that hexanal is abundant in unripe wheat grain, and as they
258 ripen rapidly drops down (Ji et al., 2017). Therefore, this compound can indicate a substrate
259 unsuitable for *T. molitor*, as the beetles successfully reproduce in the fully matured grains.

260 It is obvious that in dry wheat grain, the concentration of hexanal is too low to cause
261 repellent reaction of *T. molitor* (otherwise the grain would not be attractive). However, after mold
262 fungus infection, it is likely that its emission increases. Whether the source of hexanal is the mold
263 fungus itself or the grain it breaks down remains to be clarified in the future.

264 There was no data on behavioural or EAG response to 1-hexanol in Tenebrionidae beetles.
265 Novelty revealed in the present paper was the dual effect of this compound towards *T. molitor*
266 adults: attractivity to females and repellency (at higher concentrations only) to males. 1-Hexanol
267 was known as a repellent for grain pest *Sitophilus zeamais* (Motschulsky) *(*Coleoptera,
268 Curculionidae) (Zunino et al., 2015); however, whether it is equally effective in males and females
269 has not been investigated.

270 3-Methyl-1-butanol was revealed as an attractant for *T. molitor* females but not to males
271 for the first time. The attractant was not highly effective because it acted at high concentrations

only. This compound was known as both an attractant (at low concentrations) and repellent (at high concentrations) for adults of maize weevil, *S. zeamais* (Zunino et al., 2015), and as an attractant for larvae of red flour beetle, *T. castaneum* (Sinha, 1990).

The remaining three compounds tested, namely 1-pentanol, toluene, and 2-methylfuran, evoked no behavioural responses in either males or females of *T. molitor*. 1-Pentanol was repellent for maize weevil, *S. zeamais* (Zunino et al., 2015) and attractant at low concentrations, as well as repellent at high concentrations for wheat weevil, *S. granarius* (Germinara et al., 2008). The toluene effect was studied on two species from the genus *Tribolium*: *T. castaneum* and *Tribolium confusum* Jacquelin du Val (Coleoptera, Tenebrionidae). In the presence of toluene in the air, Pajaro-Castro et al. (2019) documented significant suppression of locomotory activity and, in some cases, mortality of *T. castaneum* beetles. No effect was revealed in *T. confusum* beetles (Titov, Brygadyrenko, 2021). 2-Methylfuran evoked no behavioural effect on *T. molitor* adults. The studies on this compound's behavioural effects on any other species of beetles, known as pests of warehouses, were not known to us.

In general, the obtained results demonstrate that wheat grain infected by the mold fungus *A. flavus* repelled *T. molitor* beetles, with females being more susceptible and males less so. The tested volatile compounds released by the grain-fungus complex have varying effects on *T. molitor* adults and depend on their sex. Among the VOCs, three were found to repel males, while one repelled and two attracted females. Changes in the proportion of these volatile components within the natural blend can significantly modify the beetles' response to infected grain. Hexanal was evaluated as a repellent, suitable for further studies under grain or crop storage conditions to reveal its potential for practical applications taking into account not only the results of this study but also the fact that the compound possesses antibacterial properties (Fan et al., 2023; Fen et al., 2025).

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Table 1. Chemical volatiles released from wheat grains infected by *Aspergillus flavus*: most often recorded in research papers.

Chemical compound	Number of reports
1-Octen-3-ol	4[1,2,3,7]
Hexanal	4[1,2,4,5]
1-Hexanol	3[1,2,3]
1-Pentanol	3[1,2,4]
Toluene	3[1,2,5]
3-Methylbutanol	3[3,6,7]
2-Methylfuran	3[1,2,3]

[1] – Duan et al., 2021; [2] – Kang et al., 2022; [3] – Magan, Evans, 2000; [4] – Lin et al., 2023; [5] – Chen et al., 2024; [6] – Kaminski al., 1976; [7] – Börjessonet al., 1989.

Table 2. Behavioural reactions of male and female *Tenebrio molitor* to volatile organic compounds from wheat grain infected by *Aspergillus flavus*: repellent effect (R), attractive effect (A), dash means no effect.

Male			Chemical compound	Female		
Concentration, mM				Concentration mM		
1	10	100		1	10	100
R	R	R	1-Octen-3-ol	-	-	-
-	R	R	Hexanal	R	R	R
-	-	R	1-Hexanol	A	A	A
-	-	-	1-Pentanol	-	-	-
-	-	-	Toluene	-	-	-
-	-	-	3-Methylbutan-1-ol	-	-	A
-	-	-	2-Methyl-furan	-	-	-

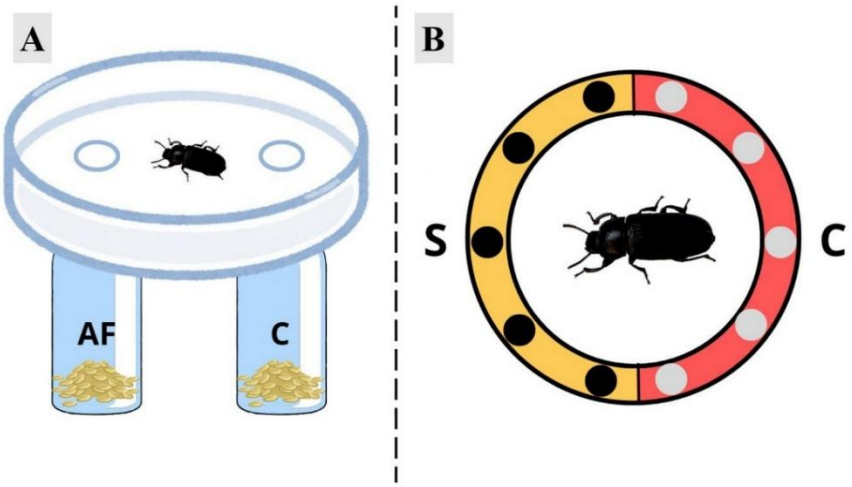

Figure 1. The principal scheme of a two-choice test: A – pitfall; B – in a Petri plate. AF – *Aspergillus flavus* infected wheat grain; C – control wheat grain or control zone; S – stimulus zone. Black circles indicate spots where a single chemical compound was applied; gray circles indicate spots of solvent drops

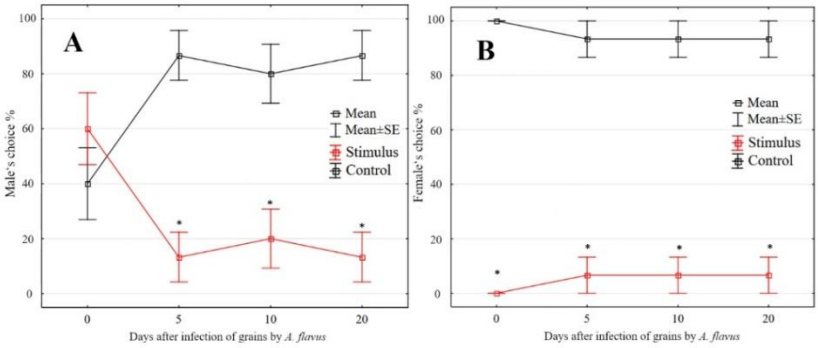

Figure 2. Choice of *Tenebrio molitor* adults (A – male and B – female) between *Aspergillus flavus*-infected wheat (stimulus) and uninfected wheat (control). Statistically significant differences in the stimulus and control are marked with an asterisk ($p<0.05$) (exact binomial test)

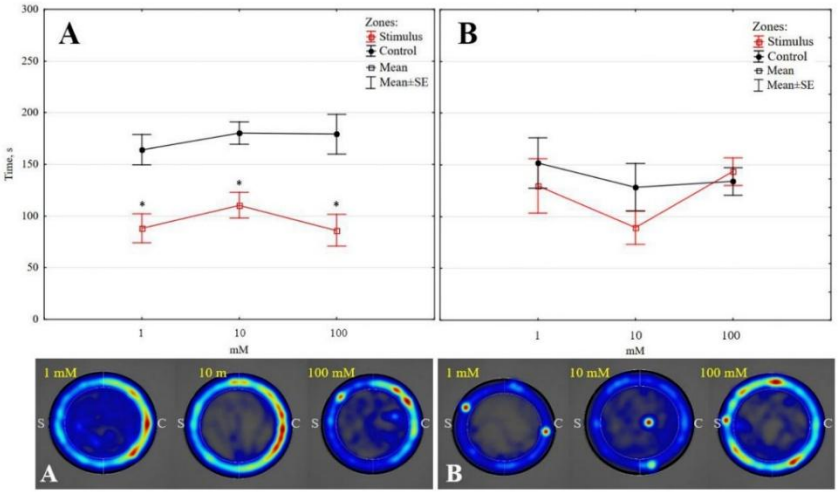

Figure 3. Average time spent by *Tenebrio molitor* beetles in alternative zones: stimulus (S) and control (C), when exposed to different concentrations of 1-octen-3-ol (n = 10, recording duration 5 min): A – male; B – female. Statistically significant differences are marked with an asterisk ($p<0.05$) (Wilcoxon matched pairs test). The lower panel shows a heat map in which a color gradient represents the cumulative time spent in the arena by 10 *T. molitor* individuals (10 × 5 min). Red indicates the longest time spent, and blue the shortest.

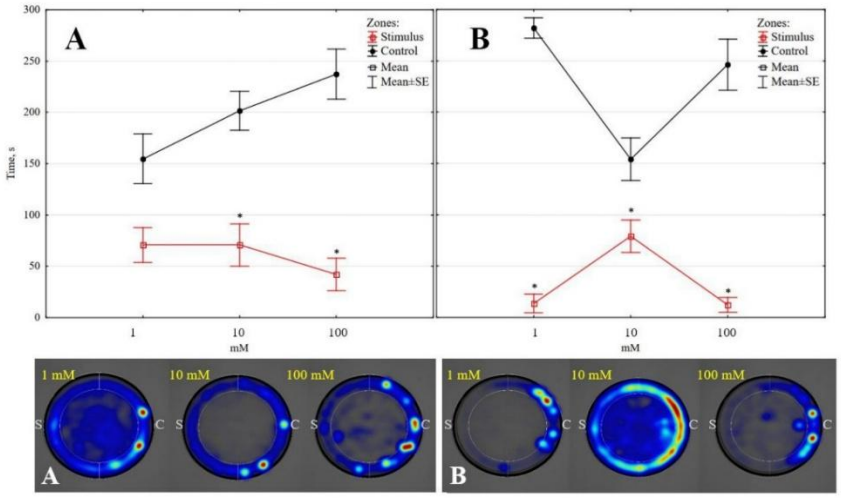

Figure 4. Average time spent by *Tenebrio molitor* beetles in alternative zones: stimulus (S) and control (C), when exposed to different concentrations of hexanal (n = 10, recording duration 5 min): A – male; B – female. Statistically significant differences are marked with an asterisk ($p<0.05$) (Wilcoxon matched pairs test). The lower panel shows a heat map in which a color gradient represents the cumulative time spent in the arena by 10 *T. molitor* individuals (10 × 5 min). Red indicates the longest time spent, and blue the shortest.

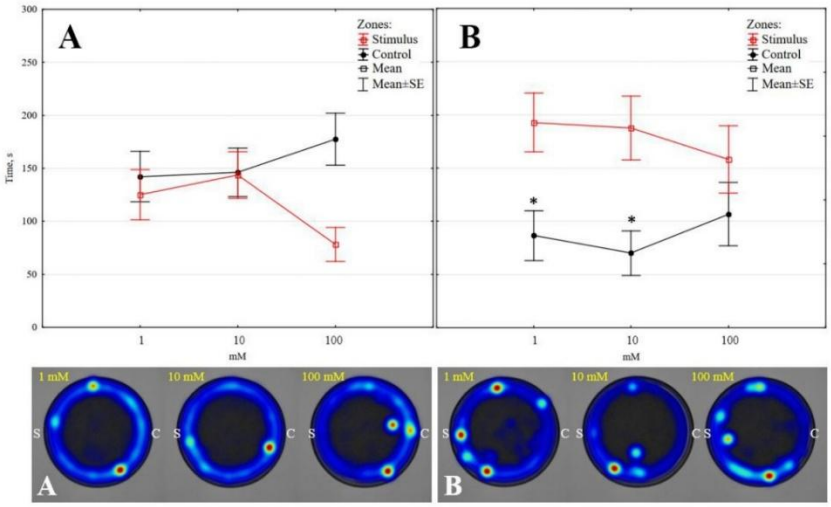

Figure 5. Average time spent by *Tenebrio molitor* beetles in alternative zones: stimulus (S) and control (C), when exposed to different concentrations of 1-hexanol (n = 10, recording duration 5 min): A – male; B – female. Statistically significant differences are marked with an asterisk ($p<0.05$) (Wilcoxon matched pairs test). The lower panel shows a heat map in which a color gradient represents the cumulative time spent in the arena by 10 *T. molitor* individuals (10 × 5 min). Red indicates the longest time spent, and blue the shortest.

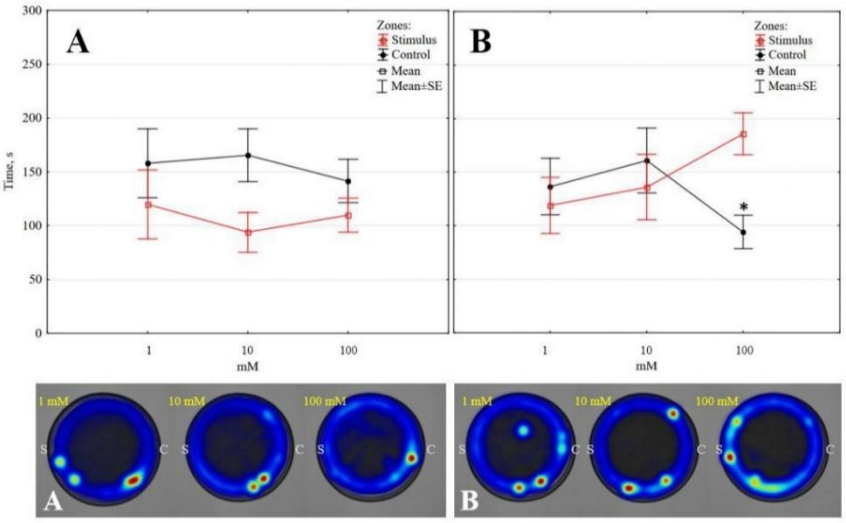

Figure 6. Average time spent by *Tenebrio molitor* beetles in alternative zones: stimulus (S) and control (C), when exposed to different concentrations of 3-methylbutanol (n = 10, recording duration 5 min): A – male; B – female. Statistically significant differences are marked with an asterisk ($p<0.05$) (Wilcoxon matched pairs test). The lower panel shows a heat map in which a color gradient represents the cumulative time spent in the arena by 10 *T. molitor* individuals (10 × 5 min). Red indicates the longest time spent, and blue the shortest.

NOTES

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Vilniaus universiteto leidykla
Saulėtekio al. 9, III rūmai, LT-10222 Vilnius
El. p. info@leidykla.vu.lt, www.leidykla.vu.lt
bookshop.vu.lt, journals.vu.lt
Tiražas 20 egz.