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ORIGINAL ARTICLE

The defensive secretion of *Eurycantha calcarata* (Phasmida: Lonchodidae) – chemical composition and method of collection

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Abstract. Chemical defence in insects is an increasingly popular subject of research and has the potential for providing unexplored compounds with unknown properties for drug and repellent discovery, so the secretions of various species of insects are currently being studied, and new ways of collecting these secretions are being sought. Silica gel and activated carbon were used as absorbents to collect the sprayed defensive secretion of *Eurycantha calcarata*. Using gas chromatography coupled with mass spectrometry, 49 compounds were identified, including 19 carboxylic acids, nine esters, ten alcohols, five hydrocarbons, and other organic compounds. The most abundant two compounds from each group were: hexadecanoic acid, octadecanoic acid, 9-hexadecenoic acid octadecyl ester, hexadecanoic acid tetradecyl ester, octacosanol, triacontanol, tridecane, and tetradecane. Silica gel turned out to be a better absorbent because it captured more compounds than the activated carbon. The mass of the absorbent did not affect the quality of the analyses. This paper is the first describing the volatile secretions emitted by phasmid representatives that originate from abdominal structures rather than the glands on prothorax. The presented results of the analyses and the known properties of the detected compounds give grounds for the conclusion that these secretions are of importance for defence in this species of phasmid.

INTRODUCTION

Insects usually use mimicry, catalepsy, or actively attack with their legs, stingers, or mouth apparatus to defend themselves (Sugiura, 2020), but some species use chemical defences to scare away or discourage predators. These insects secrete irritating compounds from specialized structures in a gaseous (Moraes et al., 2008) or liquid form (Dossey et al., 2009). Representatives of the order Phasmida use chemical defence to deter predators, although their primary defence mechanism is to resemble their surroundings. Insects from this order are dorsoventrally flattened or have a cylindrical stick-like shape, with an elongated or flattened body often covered with appendages resembling plant parts, such as leaves, branches, or bark, and having the same colour and structure (Bedford, 1978; Bian et al., 2016). If a predator manages to spot them and decides to attack, some species defend themselves by secreting irritating compounds from their glands on prothorax to scare the predator away (Dossey, 2010). One such Phasmida species that employs chemical defence is *Eurycantha calcarata*, also known as the giant spiny stick insect, which inhabits New Guinea, the Solomon Islands, and the Bismarck Archipelago (Gibson et al., 2012). This species, where the females are larger than the males, can reach up to 14 centimetres in length, and the adults of both sexes are dark brown and covered with spines. Usually, *E. calcarata* avoids predators by blending into the tree bark where they spend most of the day, but when the males are irritated or feel threatened, they raise their third pair of legs, which are armed with a sharp spike about one centimetre long, bend their abdomen in the shape of a letter S, and release a defensive secretion from undescribed abdominal structures (Boisseau et al., 2020).

The defensive secretion of this insect has not been studied and described so far, and thus, such studies have the potential to find new compounds with unknown properties. Such secretions sometimes lead to the discovery of new drugs (Dossey, 2010) and also of novel compounds with unknown effects, as confirmed by the studies mentioned below. Parectadial, (4S)-(3-oxoprop-1-en-2-yl)cyclohex-

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1-enecarbaldehyde, for example, was a new unexplored compound found by Dossey et al. (2007). It was a main component of the defensive secretion of *Parectatosoma mocquerysi* and, from the author's observations, it appears to cause a specific reaction on human skin. After contact, the skin reddens and, with greater exposure, exfoliates without any pain or itching being observed (Dossey et al., 2007). 4-methyl-1-hepten-3-one was the main compound found in the defensive secretions of *Agathemera elegans*, a species that is recognized and avoided in Chile because contact with it's secretions can cause temporary blindness due to acute conjunctivitis and corneal damage. This was the first report on the natural origin of these compounds (Schmeda-Hirschmann, 2006). Not only have the chemical composition of the defensive secretions of phasmid representatives been studied, but also the properties of the compounds contained in them. Another defensive compound from phasmid secretions with confirmed repellent properties is the iridoid actinidine, which is found in the secretion of *Megacrania tsudai*. The researchers claimed that this species stores and secretes this substance to deter predators because actinidine occurs in the host plant and has repellent properties towards both spiders and birds (Ho & Chow, 1993). Actinidine has also been found in other *Megacrania* species (Prescott et al., 2009). Quinoline, found in *Oreophoetes peruana*, repels ants, spiders, cockroaches, and frogs, and has similar properties to actinidine (Eisner et al., 1997).

The various groups of compounds contained in insect secretions can perform many functions as they are involved in chemical communication, chemical signalling, defence, and reproduction. For example, hydrocarbons are one of the better known groups of compounds in insects, and they can perform all the aforementioned functions (Howard & Blomquist, 1982; Rivault et al., 2002; Belenioti et al., 2022). In insect defensive secretions, various groups of compounds have been found, such as carboxylic acids, alcohols, esters, carbohydrates, aldehydes, among others (Laurent et al., 2004; Reitz et al., 2015; Hansson & Wicher, 2016). For example, in the defensive secretions of the nymphs and adults of both sexes of *Pyrrhocoris apterus*, 40 components were identified from the nymphal posterior dorsoabdominal glands and 35 from the adult metathoracic glands. Out of these identified compounds were 23 aldehydes, five saturated hydrocarbons, five alcohols, three ketones, three lactones, two terpenes, one phenol, and one ester (Farine, 1992).

Given that new and unexplored compounds can be found in the defensive secretions of insects, this field has become increasingly popular for potential drug and repellent discovery (Dossey, 2010). Consequently, novel methods for collecting these secreted compounds are being sought. When the secretion is in liquid form, sampling appears to be easy as it only needs to be collected in the necessary volume for analysis (Dossey et al., 2009). The problem begins when the secreted irritants are in a volatile form, when the contents of the mixtures are unknown, or when the level of analytes are insufficient to be detected by the available analytical techniques.

Studies on insect volatile defensive secretions have described various collection methods. Villaverde et al. (2007) placed insects in a glass vial with a rubber septum and, after a period of time, harvested the volatiles by sampling from the head space, followed by SPME-CG analysis. Volatile compounds from the defensive spray of *Megacrania tsudai*, induced by handling, were collected with a gastight microsyringe and directly injected in a GC-MS for analysis (Ho & Chow, 1993). In another case described by Gunbilig (2009), the whole insect was enclosed in a glass container, and the emitted volatiles were deposited onto charcoal traps while air was circulated for 24 h. The volatiles were then eluted with dichloromethane containing n-bromodecane as an internal standard and analysed by GC-MS. For males of *Eurycantha calcarata*, however, a new sampling method was created due to it's large size and need for manual irritation in order to release its secretion, thus making the previous methods inappropriate. The insect was irritated in a terrarium, where secretions were more likely to be released and their strong characteristic smell detected. Sorbents were then used to collect the secretions.

No further research has thus far been conducted on the secretions of *Eurycantha calcarata*, however, based on it's observed behaviour and noticeable smell when irritated, it can be assumed that it uses chemical compounds to deter predators. Glands on prothorax are not found in this species, so it is assumed that the secretion is related to the structures located at the end of the abdomen. This secretion contains compounds that have a repellent and irritating effect, as the intense odour appears when the insects are threatened. This type of sample has not been collected from living individuals so far, so it was decided to use two types of absorbents in order to capture the volatile odour compounds, each having the ability to capture different compounds.

In this study, the chemical composition of the defensive secretion of male *E. calcarata* was analysed, and the absorbing properties of silica gel and activated carbon were compared to see if there were any differences in the chemical analyses in terms of the number and quantity of detected compounds. This is the first description of the chemical composition of the defensive secretion of *E. calcarata*. Additionally, the method of collection using silica gel and activated carbon for the extraction of the defensive secretion in sprayed form in phasmids was used for the first time.

MATERIAL AND METHODS

Eurycantha calcarata

The insects used for the analyses were obtained from breeding that began in 2019, with the third generation of males hatched in 2022 being used for the study. Eggs were from sexual reproduction and were kept in a glass incubator at a temperature of 21–25°C and 75–85% humidity until hatched. Young individuals were transferred to a glass terrarium with coconut peat, a glass bowl with fresh water, and food plants. From the moment of hatching, the insects were fed with ivy (*Hedera helix* it is a com-

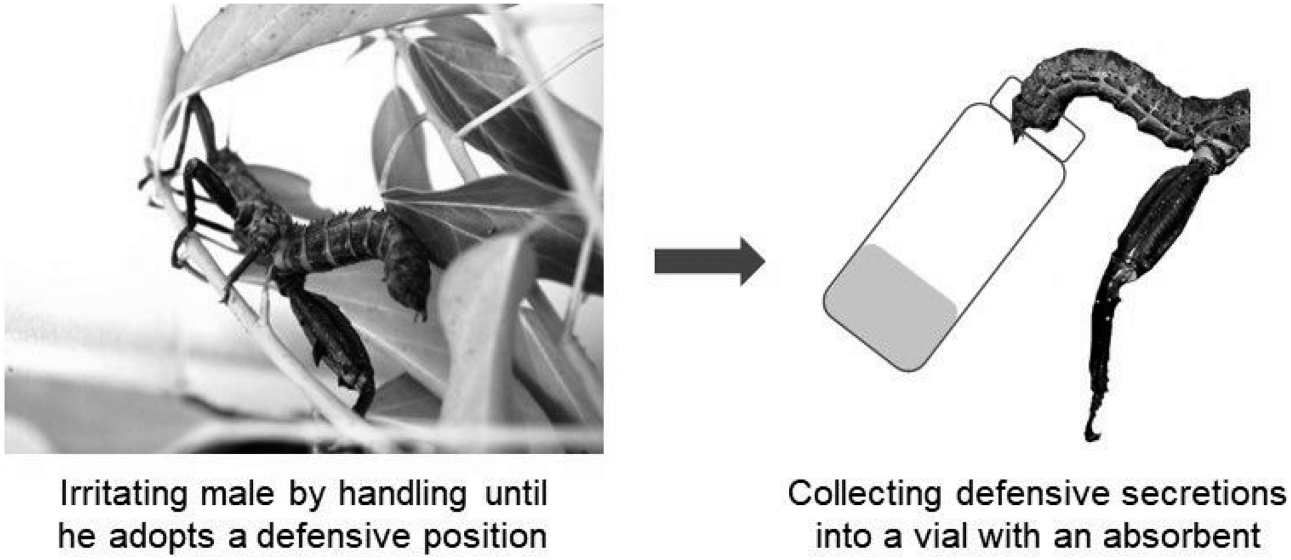

Fig. 1. Method of collecting defensive secretions.

mon species not under protection, and its collection did not require permits) and kept at a temperature of 21–25°C temperature with a humidity of 70–85% until they reached maturity.

Chemicals and reagents

The following standards were used in the analyses: nonanoic acid (C9:0) (Merck), dodecanoic acid (C12:0) (Carl Roth RG), pentadecanoic acid (C15:0) (Merck), hexadecanoic acid (C16:0) (Merck), eicosanoic acid (C20:0) (Fluka AG), tetracosanoic acid (C24:0) (Merck), 2-hydroxypropionic acid (Merck), adipic acid (Merck), azelaic acid (Merck), dodecanoic acid, 1-methylethyl ester (Merck), tetradecanoic acid, 1-methylethyl ester (Merck), tetradecanoic acid, 2,3-dihydroxypropyl ester (Merck), hexadecanoic acid octadecyl ester (Merck), glycerol (Merck), 1-monopalmitoylglycerol (Merck), 1-monooleoylglycerol (Merck), 1-monostearin (Merck), dodecanol (Merck), eicosanol (Merck), triacontanol (Merck), and n-alkane standards (Polyscience corporation).

Sample preparation and chemical analysis

Bedford (1975) mentioned that a strong-smelling fluid from unknown structures is associated with the male's copulating block. However, this observation has not been confirmed by other researchers. There are no histological and anatomical reports regarding this structure in *E. calcarata*. To verify if the secretion is released from the abdominal structures, a vial was placed at the apex of the abdomen while the male *E. calcarata* was irritated.

The defensive secretion from the adult male was collected by placing the end of the insect's abdomen at irritation time into the vial with the absorbent (Fig. 1); when the characteristic smell was perceptible, the insect then deposited the secretion into the vial. Each sample consisted of three deposits by randomly selected insects from the terrarium. In order to determine which chemical compounds were in the terrarium, a vial containing 1 gram of silica gel was put into the terrarium and left there for exactly the same amount of time that it took for the samples to be taken from the insects (8 min).

To obtain a filtrate for further analysis, the absorbents had to be rinsed with dichloromethane. In order for the extract to be as concentrated as possible, it was necessary to determine what the smallest amount of dichloromethane needed to rinse the absorbent to obtain the filtrate would be. Various amounts of dichloromethane were therefore added to 1 and 2 grams of the silica gel and activated carbon, and it was found that for 1 gram of silica gel, 3 ml of CH_2Cl_2 should be added, for 2 g of this absorbent 5 ml, for 1 g of activated carbon 4.5 ml, and for 2 g 9 ml. After shaking for two minutes, the solution from each vial was passed through filter paper, and the extracts poured into smaller vials. In order to check which extraction method would prove to be the most effective in yielding the most compounds, four silica gel extracts were prepared for analysis in different ways. To minimize the risk of evaporation of the more volatile compounds, the extracts from 1 and 2 g of silica gel were evaporated in the air and analysed. The other two extracts were evaporated under nitrogen. All the extracts were subjected to the derivatization process. A mixture of 100 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide ($CF_3C[=NSi(CH_3)_3]OSi(CH_3)_3$) was added to each sample, the latter weighing approximately 1 mg. The samples were heated for 1 h at 100°C.

The organic compounds in each sample were examined by gas chromatography coupled with mass spectrometry. A Shimadzu GCMS-QP2010 SE gas chromatograph coupled with a QP2010 SE (Shimadzu Corporation, Kyoto, Japan) quadrupole mass detector was used to analyse the insect compounds. The samples were introduced to the gas chromatograph, which was equipped with a Zebron ZB-5 capillary column, 30 m × 0.25 mm × 0.25 µm (Phenomenex, USA). Helium was the carrier gas, and the process occurred over a temperature range of 60 (held for 3 min) to 310°C, increased at a rate of 4°C/min. The ion source was kept at a temperature of 200°C. The transfer line and injector were heated to 310°C. Individual compounds were identified from their electron impact mass spectral patterns. All compounds were identified by comparing the retention time of the analysed compounds with standards and also on the basis of characteristic ions. Additionally, a mass spectral library was used (Gołębiowski et al., 2016; Wojciechowska et al., 2022). Fatty acids (trimethylsilyl derivatives) were identified on the basis of their characteristic ions at *m/z* 117, 129, 132, 145, [M-15]+, and $M^{+\cdot}$ (molecular ion). Characteristic ions of alcohols (trimethylsilyl derivatives) were [M-15]+, *m/z* 73, 75, and 103. Alkanes were identified on the basis of the characteristic ions at *m/z* 43, 57, 71, 89, 99, and ($M^{+\cdot}$). The mass spectrum of the trimethylsilyl ester of 2-hydroxypropionic acid showed the following ions: [M-15]+ (219), and fragment ions at *m/z* 73, 117, 147, and 191. The mass spectrum of the trimethylsilyl ester of adipic acid showed the following

Table 1. Characteristic ions of the esters and compounds from other groups.

Compound	MW	Characteristic ions (m/z)
Dodecanoic acid 1-methylethyl ester (Isopropyl laurate)	242	41, 43, 57, 60, 73, 102, 129, 143, 157, 183, 200, 242
2,2,4-trimethyl-3-carboxy isopropyl pentanoic acid isobutyl ester	286	43, 55, 71, 97, 111, 143, 159, 243
Tetradecanoic acid, 1-methylethyl ester (Isopropyl myristate)	270	41, 43, 57, 60, 102, 129, 185, 211, 228, 270
Tetradecanoic acid, 2,3-dihydroxypropyl ester (TMS)	446	73, 147,211, 343, 431
Hexadecanoic acid tetradecyl ester	452	43, 57, 71, 83, 97, 111, 196, 257, 452
9-hexadecenoic acid octadecyl ester	506	43, 57, 69, 83, 97, 111, 236, 255, 506
Hexadecanoic acid octadecyl ester	508	43, 57, 71, 83, 97, 111, 257, 508
Octadecanoic acid, octadecyl ester	536	43, 57, 69, 83, 97, 111, 285, 536
9-hexadecenoic acid, eicosyl ester	534	57, 69, 83, 97, 152, 194, 236, 255, 534
Glycerol (TMS)	308	73, 103, 117, 133, 147, 205, 218, 293
Manoyl oxide	290	43, 55, 69, 81, 95, 109, 123, 137, 177, 192,257, 275, 290
1-monopalmitoylglycerol (TMS)	474	73, 129, 147, 205, 239, 371, 459
1-monooleoylglycerol (TMS)	500	73, 103, 129, 147, 203, 397, 485, 500
1-monostearin (TMS)	502	73, 103, 129, 147, 399, 487
Cholesterol (TMS)	458	43, 57, 73, 129, 145, 247, 255, 329, 353, 368, 443, 458

ions: *m/z* 55, 73, 75, 111, 117, 129, 141, 185, and [M-15]+ (203). Azelaic acid was identified on the basis of the characteristic ions at *m/z* 55, 73, 75, 117, 129, 147, 201, and [M-15]+ (317). Table 1 shows the characteristic ions of the esters and compounds from other groups.

RESULTS AND DISCUSSION

49 compounds were identified from the secretions of *Eurycantha calcarata* adult males: 19 carboxylic acids, nine esters, ten alcohols, five hydrocarbons, glycerol, 1-monopalmitoylglycerol, 1-monooleoylglycerol, 1-monostearin, cholesterol, and manoyl oxide. The occurrence of compounds in the analyses differed due to the manner in which the secretions were collected and the samples extracted. Table 2 shows the sample number (used in the tables below), along with a description of the collection method, extraction method, and the number of identified compounds. Table 3 shows the number of compounds that were identified from each group per analysis.

From Tables 2 and 3, it can be concluded that the addition of a silylating agent increased the detectability of the compounds contained in the defensive secretions of this insect. On analysing the presence of organic compounds in *E. calcarata* secretions, we observed differences in the number of detected compounds depending on the absorbent used. Silica gel turned out to be a better absorbent because it captured more compounds than activated carbon. The amount of absorbent used did not affect the quality of the analyses. There were no significant differences between the occurrence of carboxylic acids among samples using the different absorbents. In samples 2–4 there were fewer identified acids. There was also no correlation between the amount of detected acids and the amount of absorbent used, suggesting that the quantity of absorbent does not affect the quality of the analyses. It was not possible to determine clear dependencies between the presence of alcohols and the amount of absorbent used. There were also no clear dependencies between the presence of esters and the amount and type of the absorbent used.

Tables 4–8 shows the identified compounds in each analysis. The most numerous group of compounds from analyses were carboxylic acids (Table 4): 19 different compounds were identified, the most abundant being hexadecanoic acid and octadecanoic acid. Carboxylic acids have been found in the defensive secretions of insects and in cuticular lipid mixtures, the latter possessing antimicrobial activity (Gołębiowski et al., 2012a, 2014; Urbanek et al., 2012). In *Carabus lefebvrei* pupae, carboxylic acids occur in the glandular secretion. The researchers claimed that the compounds (including carboxylic acids) found in the pygidial gland secretion are a deterrent against predators (Giglio et al., 2009). Hexadecanoic acid is often found in insects. This compound is a solid organic chemical compound from the fatty acid group. Excess carbohydrates are converted into hexadecanoic acid, which is a precursor to the production of longer-chain fatty acids (Heil et al., 2019). It is assumed that the high amount of hexadecanoic acid is related to its function as a precursor for the production of longer chain fatty acids, and that it may be involved in metabolic processes leading to the formation of other

Table 2. Samples of the defensive secretion of the adult male of *E. calcarata* – sample number, absorbent used, method of extraction, and number of identified compounds.

Sample number	Absorbent	Amount	Extraction method	Number of identified compounds
1	Silica gel (terrarium)	1 g	evaporation with nitrogen + silylating agent	28
2	Silica gel	1 g	evaporation in the air	6
3	Silica gel	1 g	evaporation with nitrogen	5
4	Silica gel	2 g	evaporation in the air	7
5	Silica gel	2 g	evaporation with nitrogen + silylating agent	30
6	Activated carbon	1 g	evaporation with nitrogen + silylating agent	16
7	Activated carbon	1 g	evaporation with nitrogen + silylating agent	12
8	Activated carbon	2 g	evaporation with nitrogen + silylating agent	22
9	Activated carbon	2 g	evaporation with nitrogen + silylating agent	18

Table 3. Number of identified hydrocarbons, carboxylic acids, alcohols, esters, and other groups of compounds (sample numbers from Table 2).

Sample number	Absorbent	Amount	Extraction method	Hydrocarbons	Carboxylic acids	Alcohols	Esters	Other compounds
1	Silica gel (terrarium)	1 g	evaporation with nitrogen + silylating agent	3	13	8	1	3
2	Silica gel	1 g	evaporation in the air	2	1	1	2	0
3	Silica gel	1 g	evaporation with nitrogen	1	4	0	0	0
4	Silica gel	2 g	evaporation in the air	3	1	0	2	1
5	Silica gel	2 g	evaporation with nitrogen + silylating agent	5	15	9	0	1
6	Activated carbon	1 g	evaporation with nitrogen + silylating agent	0	4	2	6	4
7	Activated carbon	1 g	evaporation with nitrogen + silylating agent	0	11	0	0	1
8	Activated carbon	2 g	evaporation with nitrogen + silylating agent	0	14	4	2	2
9	Activated carbon	2 g	evaporation with nitrogen + silylating agent	0	13	4	0	1

compounds. Octadecanoic acid, also known as stearic acid, is a white waxy solid with a mild tallow-like odour. Its esters are found in animal fats (Heil et al., 2019). It probably performs the function of storing active substances because it is a solid, and active compounds can be suspended in it. Among other insects, hexadecanoic and octadecanoic acids were present in the highest amounts compared to other compounds in the defensive secretion of the bug *Graphosoma lineatum* (Hemiptera: Pentatomidae) (Durak & Kalender, 2009).

Esters were the second most abundant group of compounds in the analytes (Table 5). 9-hexadecenoic acid octadecyl ester and hexadecanoic acid tetradecyl ester had the highest percentage content (Table 5). Little is known about the octadecyl ester of 9-hexadecenoic acid, and so far it has not been found in the defensive secretions of insects, although it's presence is recorded in *Ceratitis capitata* before and during the breeding period (Al-Khshemawee et al., 2018). Hexadecanoic acid tetradecyl ester, or myristyl palmitate, is referred to as a xenobiotic metabolite of bacterial and fungal origin. It arises from tetradecan-1-ol (Javid et al., 2020). The presence of this compound in the secretion of *Eurycantha calcarata* may indicate the presence and use of endosymbionts (bacteria or fungi) in this insect. Another possibility is that it arises as a xenometabolite from host plants and is merely accumulated by phasmids. Determining the origin and possible functions of this compound in *E. calcarata* requires further research. Octadecanoic acid octadecyl ester, 9-hexadecenoic acid eicosyl ester, tetradecanoic acid 2,3-dihydroxypropyl ester, and hexadecanoic acid octadecyl ester are compounds that are not well understood. There is no information on their occurrence in plants or animals, let along insects. The presence of these esters was found in only one analysis, but if confirmed it would be the first report of their occurrence in insects. It should be determined that these esters do not come from background impurities. Some of the other esters identified in the analyses have previously been found in insects (Wimmer et al., 2007). These include isopropyl myristate, found in three analyses. It is a polar compound used as a moisturiser in topical cosmetics and medical preparations to improve skin absorption, and has been extensively researched and used as a skin penetration enhancer (Engelbrecht et al., 2012). Among insects, it was detected in analyses of extracts from the head and body of the ant *Iridomyrmex humilis* (Cavill & Houghton, 1974) and the surface waxes of *Halobates hawaiiensis* (Tsoukatou et al., 2001). Its potential function in phasmids may be to increase the absorption of irritating substances, thus strengthening their effect on the attacker.

Table 4. Percentage content of identified carboxylic acids (sample numbers from Table 2).

Compound	1	2	3	4	5	6	7	8	9
2-hydroxypropionic acid	–	–	–	–	–	–	2.50	–	–
Hexanoic acid	0.35	–	–	–	–	–	1.91	3.83	3.38
Heptanoic acid	0.10	–	–	–	0.06	–	–	0.76	1.01
Nonanoic acid	–	3.29	0.83	1.91	2.38	–	7.61	8.29	9.40
Octanoic acid	0.68	–	–	–	0.50	–	–	3.06	–
Decanoic acid	–	–	–	–	0.21	–	0.24	0.55	0.53
Adipic acid	–	–	–	–	–	–	1.70	–	–
Dodecanoic acid	0.21	–	–	–	0.04	–	–	0.20	0.31
Azelaic acid	–	–	–	–	0.22	–	2.03	0.29	0.53
Tetradecanoic acid	0.37	–	4.91	–	0.22	–	0.77	0.69	0.94
Pentadecanoic acid	0.33	–	–	–	0.36	–	0.53	0.82	0.79
Hexadecanoic acid	11.82	–	24.92	–	10.47	8.45	35.90	28.86	34.70
Heptadecanoic acid	0.51	–	–	–	0.60	0.34	0.59	1.16	0.71
Octadecanoic acid	8.55	–	10.79	–	5.74	9.83	31.98	19.12	26.67
Eicosanoic acid	0.55	–	–	–	0.62	4.95	–	0.92	0.81
Heneicosanoic acid	0.15	–	–	–	–	–	–	–	–
Docosanoic acid	1.27	–	–	–	1.76	–	–	1.96	2.14
Tetracosanoic acid	–	–	–	–	0.44	–	–	–	–
Hexacosanoic acid	0.42	–	–	–	0.20	–	–	–	–

Table 5. Percentage content of identified esters (sample numbers from Table 2).

Compound	1	2	3	4	5	6	7	8	9
Dodecanoic acid 1-methylethyl ester (Isopropyl laurate)	–	0.15	–	0.12	–	–	–	–	–
2,2,4-trimethyl-3-carboxy isopropyl pentanoic acid isobutyl ester	0.47	–	–	–	–	–	–	1.91	–
Tetradecanoic acid, 1-methylethyl ester (Isopropyl myristate)	–	0.57	–	0.64	–	–	–	0.21	–
Tetradecanoic acid, 2,3-dihydroxypropyl ester	–	–	–	–	–	2.02	–	–	–
Hexadecanoic acid tetradecyl ester	–	–	–	–	–	5.17	–	–	–
9-hexadecenoic acid octadecyl ester	–	–	–	–	–	7.55	–	–	–
Hexadecanoic acid octadecyl ester	–	–	–	–	–	2.33	–	–	–
Octadecanoic acid, octadecyl ester	–	–	–	–	–	4.69	–	–	–
9-hexadecenoic acid eicosyl ester	–	–	–	–	–	4.90	–	–	–

The two most abundant alcohols were octacosanol and triacontanol (Table 6). Researchers describe the alcohols found in insect defence secretions as compounds found in smaller amounts, and they do not assign them any great defensive role; however, some insects use alcohols for defence, although indirectly. The larvae of many beetles in the family Chrysomelidae sequester phenolglucosides, such as salicin, from their food plants, such as Salix and Populus spp. Salicin is hydrolysed in the glandular reservoir of the defence glands. The resulting salicylic alcohol (saligenin) is oxidized by extracellular oxidase. The product, salicylaldehyde, accumulates as the main defence compound of these beetles (Brückmann et al., 2002). It seems that alcohols may be used in a similar way by *Eurycantha calcarata*, but further research is required to determine whether they are only accumulated or are subject to further metabolic transformations. Octacosanol is a straight-chain aliphatic fatty alcohol that is used as a nutritional supplement. This organic compound is the main component of a natural wax extracted from plants. Octacosanol is reported to possess cholesterol-lowering effects, antiaggregatory properties, cytoprotective use, and ergogenic properties. It has been studied as a potential therapeutic agent for the treatment of Parkinson's disease (Wang et al., 2010). Triacontanol is an ultra-long-chain primary fatty alcohol that has been shown to significantly inhibit the feeding activity of insects *Spilosoma obliqua* and *Spodoptera litura* (Pande et al., 1995). Emam et al. (2018) investigated the effect of treating Schefflera plants with the hormone triacontanol (TRIA). These studies showed that plants treated with two concentrations of this compound suffered reduced herbivory when exposed to *Macrosiphum euphorbiae* infestation compared to the control. It is possible that insects use it as a deterrent.

Hydrocarbons have been identified in many insect species (Lockey, 1988; Gołębiowski, 2012b). They are used to protect insects from desiccation and serve as pheromones. In our study, the hydrocarbons occurring in the largest amounts were tridecane and tetradecane (Table 7); although these two compounds were present in the control sample, they were more than twice as high in the defensive secretion samples (Table 7). It is assumed that in a closed terrarium where insects are living, volatile compounds emitted by them will also be present in the atmosphere of the insectarium. Tridecane is a straight-chain alkane with 13 carbon atoms, and it is a component of essential oils isolated from plants (Magwa et al., 2006). In its pure form it is an oily yellow transparent liquid with a characteristic smell. Repeated or prolonged contact of tridecane with the skin may irritate and cause redness, leading to inflammation. Exposure to high vapor concentrations can cause headache and stupor (Muhammad, 2005; Magwa et al., 2006). In insects, tridecane occurs in the volatile secretion of the bug *Cosmopepla lintneriana* (Hemiptera: Pentatomidae). The Pentatomidae family are known for using volatile odorants as a predator deterrent, which they release when threatened. The secretion of this bug, consisting mainly of tridecane, acts as a deterrent to birds and lizards (Krall et al., 1999; Guadalupe et al., 2019). Tridecane was also found in *Graphosoma lineatum* secretions. It has been identified as a toxic, irritant, or repellent compound, which is released by these bugs in response to irritation and which also acts as a predator deterrent (Durak & Kalender, 2009). This alkane was also found in the secretion from the metathoracal glands of another Pentatomide species, *Piezodorus guildinii*, where it performed the same functions. Tridecane is known as a toxin, irritant or repellent released by Pentatomidae in response to irritation. It suggests that this compound acts as a chemical defence for these species

Table 6. Percentage content of identified alcohols (sample numbers from Table 2).

Compound	1	2	3	4	5	6	7	8	9
Dodecanol	0.21	0.88	–	–	0.22	–	–	0.33	0.22
Tetradecanol	0.14	–	–	–	0.21	–	–	–	–
Hexadecanol	0.33	–	–	–	0.37	0.16	–	0.63	0.42
Oktadecanol	–	–	–	–	0.29	2.58	–	–	–
Eicosanol	0.06	–	–	–	–	–	–	–	–
Docosanol	0.47	–	–	–	0.59	–	–	–	–
Hexacosanol	0.78	–	–	–	1.37	–	–	–	–
Octacosanol	8.97	–	–	–	12.77	–	–	10.64	8.21
Triacontanol	5.98	–	–	–	8.38	–	–	5.99	3.62
Dotriacontanol	–	–	–	–	2.24	–	–	–	–

Table 7. Percentage content of identified hydrocarbons (sample numbers from Table 2).

Compound	1	2	3	4	5	6	7	8	9
Dodecane	0.68	–	–	1.76	0.83	–	–	–	–
Tridecane	0.93	2.62	–	2.95	1.33	–	–	–	–
Tetradecane	21.71	59.86	23.39	66.05	34.14	–	–	–	–
Heptacosane	–	–	–	–	0.47	–	–	–	–
Hentriaceontane	–	–	–	–	0.97	–	–	–	–

(Zarbin et al., 2000). Tridecane was present in both sexes in the defensive secretion of *Parastizopus transgariepinus* (Geiselhardt et al., 2007). Experiments on the reaction of this beetle to the chemical showed that tridecane was attractive to females but not males, which indicates that tridecane could also be a sex pheromone.

Tetradecane is a straight-chain alkane consisting of 14 carbon atoms. It is a natural compound found in plants in essential oils, known to be a secondary plant metabolite (Wang et al., 2020). Interestingly, it has recently been shown (Pan et al., 2021) that the amount of volatile tetradecane in maize roots increases after the invasion of the larvae of the beetle *Holotrichia parallela* (Melolonthidae). These studies show that tetradecane can act as a signal to induce defences and prepare plants for the following attacks, including causing metabolic changes (Pan et al., 2021). It was also shown that the growth of *H. parallela* larvae was inhibited when fed with maize roots after exposure to tetradecane. This compound can be extracted from plants and used by *E. calcarata* as a compound to deter potential predators. There is also the possibility that tetradecane is being used as a signal of impending danger to other individuals.

Among "other groups of compounds" (Table 8), the following compounds were present: glycerol, 1-monopalmitoylglycerol, 1-monooleoylglycerol, 1-monostearin, cholesterol, and manoyl oxide. Manoyl oxide is a natural product found in plants. For example, this compound was present in *Juniperus macrocarpa* and *Juniperus oxycedrus* (Meringolo et al., 2022). Glycerol in insects prevents their tissues from freezing, its accumulation being induced by decreasing temperatures (Urbanek et al., 2012). Triglycerides are typical components of the body fat of humans, along with other animals (including insects) and plants. They store energy, while also providing defence against harmful factors (Lockey, 1988; Buckner, 1993; Gołębiowski et al., 2014). Cholesterol is the most commonly found steroid in insect lipids. The cholesterol found in insects may originate from their food, and insects also convert phytosterols to cholesterol (Buckner 1993; Gołębiowski et al., 2011). This compound is an important component of cell membranes and a precursor of ecdysone.

CONCLUSIONS

This paper is the first to describe the volatile secretions produced and emitted by representatives of Phasmida that do not originate from the glands on prothorax. For the first time, absorbents such as silica gel and activated carbon were used to collect defensive secretions in a sprayed form from phasmid species, which were irritated by handling. Silica gel turned out to be more effective in detecting organic compounds than activated carbon. The addition of a silylating agent facilitated the chromatographic analysis as the compounds became more volatile, and the chromatographic signals were better resolved. Gentler evaporation of the solvent did not significantly affect the quality of the analyses because highly volatile compounds were not detected in the analyses of extracts evaporated in the air.

The presented results and the discovery of known compounds with proven irritating effects, such as tridecane, allows us to conclude that this insect uses the structures at the end of the abdomen to chemically deter predators. Research on the chemistry of phasmids may also provide new insights into their phylogenetic relationships. Limonene – a compound with a structure similar to parectadial (found in the secretion of *Parectatosoma mocquerysi*) – was also found in *Sipyloidea sipylus*, an unrelated stick insect from Southeast Asia. Researchers speculate that similarities in the chemical composition of the secretions of these insects may indicate the relationship of species from different taxonomic groups and shed new light on the evolution of these insects (Dossey et al., 2007).

Compounds from the defensive secretions of phasmid representatives have a described deterrent effect on various groups of animals. For example, tridecane, found in the defence secretion of *E. calcarata*, has been described as an irritating repellent compound. In the era of searching for new methods of plant protection that do not have a negative impact on the environment, compounds found in nature that have a deterrent effect on common crop pests, such as insects, birds, and snails, are being sought. Tridecane and tetradecane are compounds found in plants as secondary metabolites that could be used as plant protection products due to their deterrent effect and mobilizing the plant

Table 8. Percentage content of identified compounds from other groups of compounds (sample numbers from Table 2).

Compound	1	2	3	4	5	6	7	8	9
Glycerol	6.11	–	–	–	3.63	0.48	14.24	3.34	4.41
Manoyl oxide	–	–	–	0.54	–	–	–	–	–
Monopalmitate	1.36	–	–	–	–	9.81	–	–	–
Monooleate	–	–	–	–	–	2.82	–	–	–
Monostearin	0.16	–	–	–	–	–	–	–	–
Cholesterol	–	–	–	–	–	21.44	–	–	–

response to invasion (Pan et al., 2021). Further research should be conducted on the potential of using compounds from insect defence secretions in plant protection products and on how they impact the insects (Wojciechowska et al., 2022).

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