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Research Article

The Tergal Gland Secretion of the Two Rare Myrmecophilous Species *Zyras collaris* and *Z. haworthi* (Coleoptera: Staphylinidae) and the Effect on *Lasius fuliginosus*

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The beetle species *Zyras collaris* and *Z. haworthi* belong to the rove beetle tribe Myrmedoniini (Staphylinidae: Aleocharinae), which comprises many myrmecophilous species. Due to their rareness, it is unknown how the two species interact with their host ants. GC-MS analyses revealed that both species release α-pinene, β-pinene, myrcene and limonene from their defensive tergal glands. This composition of tergal gland secretion is unique within the subfamily Aleocharinae. In biotests, *Lasius fuliginosus* ants showed increased antennation towards filter paper balls treated with mixtures of these substances in natural concentrations. Because these monoterpenes are also present in some aphid species which are attended by ants, we hypothesize that *Zyras* beetles mimic the presence of aphids and thereby achieve acceptance by their host ants.

1. Introduction

The rove beetles tribe Myrmedoniini (Staphylinidae: Aleocharinae) contains many myrmecophilous species. In Central Europe, it comprises the myrmecophilous genera *Lomechusa* and *Lomechusoides, Zyras, Myrmoecia,* and *Pella*, as well as the nonmyrmecophilous species *Drusilla canaliculata* Fabricius, 1787. *Myrmoecia* and *Pella* were formerly considered subgenera of *Zyras* but, meanwhile, have been elevated to genus rank [1–3], which is also supported by molecular data [4, 5].

Lomechusa and *Lomechusoides* are textbook examples for the integration of myrmecophiles in ant nests by the use of appeasement glands on their abdomen [6]. Different strategies are used by *Pella* species to escape from aggressions by their host ant *Lasius fuliginosus* (Latreille, 1798). While the Japanese species *P. comes* (Sharp, 1874) mimics the cuticular hydrocarbon (CHC) pattern of its host ant to be accepted [7], *P. laticollis* (Märkel, 1845) employs a specific appeasing behaviour [8]. *Pella cognata* (Märkel, 1842), *P. funesta* (Gravenhorst, 1806), and *P. humeralis* (Gravenhorst, 1802) repel ants by the use of their abdominal tergal gland. This tergal gland is only found within the Aleocharinae and is used by most species of the subfamily as defensive gland against aggressors [9]. In *P. funesta* and *P. humeralis,* the gland secretion specifically contains sulcatone, a panic alarm inducing pheromone of *L. fuliginosus.* By the release of this compound, beetles create an "ant free space" [8, 10]. In contrast to these species, only little is known on the biology of *Zyras* species, and it is unclear how they achieve acceptance by ants. For *Z. collaris* (Paykull, 1789) and *Z. haworthi* (Stephens, 1835), this is mainly due to their rarity. For South-West Germany, only 18 and 10 records exist from 1950 to 2000 for *Z. collaris* and *Z. haworthi*, respectively [11]. Our own collection efforts between 2001 and 2011 resulted in approximately 1200 specimens of different *Pella* species, but only one for each of the two *Zyras* species.

Here we report for the first time on the composition of the tergal gland secretion of *Z. collaris* and *Z. haworthi* and its potential role for the interaction with its putative host ant *L.*

Table 1: Substances found in the headspace of a flask containing rove beetles of the genus *Zyras,* which have been teased using a magnetic stir bar. Numbers in the table refer to numbers in Figure 1. Relative proportions of the substances between the beetles were calculated in accordance with [12]. The substance with the highest peak area for each row is the reference (= 1.00).

		Z. collaris		*Z. haworthi*	
	Substances	Rel. peak area	Rel. proportion	Rel. peak area	Rel. proportion
1[1]	α-pinene[2]	2.6	0.20	23.8	1.00
2	β-pinene[3]	41.3	1.00	57.0	0.76
3	Myrcene	51.9	1.00	13.6	0.14
4	Limonene	4.2	1.00	5.5	0.72

[1]Numbers refer to numbers in Figure 1.
[2,3]As proposed by the mass spectra database (see Section 2).

fuliginosus. Because the study is based on the analysis of only two *Zyras* specimen, more studies with these rare beetles are urgently needed to substantiate our findings.

2. Materials and Methods

2.1. Insects. One specimen of *Z. collaris* and one of *Z. haworthi* were collected in the state of Baden-Württemberg (Germany), the first in neglected grassland near Freiburg and the second in a rural area near Herrenberg, in the vicinity of a nest of *L. fuliginosus*. The nest was located in a stump between hedgerows along a brook.

In the lab, beetles were kept in plastic Petri dishes (diameter 90 mm) at room temperature under daylight conditions. The Petri dishes were filled with a 5 mm plaster layer, which was moistened daily to maintain humidity. A small piece of filter paper was provided as shelter. Beetles were fed with dead workers of *L. fuliginosus*. Ants used as food for the beetles and for behavioural observations were collected along ant trails near the nest entrances in the vicinity of Stuttgart (State of Baden-Württemberg, Germany). Insects were determined to species level using the identification keys by Lohse [13] for beetles and Seifert [14] for ants.

2.2. Chemical Analysis of the Tergal Gland Secretion. Volatiles released from the defensive tergal glands of the beetles were analysed as described in [10]. Beetles were placed in a flask and teased with a magnetic stir bar and a magnetic stick. The volatiles from the headspace of the flask were collected using a SPME-fiber coated with 65 μm Polydimethylsiloxane/Divinylbenzene [15]. The SPME-fiber was inserted into a gas chromatograph (Type 6890; Agilent Technologies, HP 5 column: 30 m long, 0.2 mm in diameter and 0.5 μm film thickness; splitless mode, programmed: 60°C for 3 min, 60°C to 300°C at 3°C/min and then constant over 30 min at 300°C, carrier gas: Helium 1.6 mL/min) coupled to a 5973 network mass selective detector (GC-MS) for identification of the collected substances. Chromatograms and mass spectra were analyzed with Agilent Technologies software (Enhanced Chemstation MSD Chem Station D 01.02.16, June 15, 2002) using Wiley- (Wiley275) and NIST-databases (NIST Mass Spectral Library 2002 Version). For identification, mass spectra and retention times of substances were compared with respective data from synthetic compounds.

2.3. Experiments on the Effect of the Tergal Gland Secretion. Ten *L. fuliginosus* ants were placed in a Petri dish with a filter paper ball in the center. The filter paper ball was treated with 10 μL terpene solution in hexane, containing a mixture of monoterpenes in a total concentration of either 1 μg/μL or 10 μg/μL. Control filter paper balls were treated with 10 μL hexane. Each test solution was tested 20 times with different ant specimen. Hexan as control was tested 40 times. The reaction of the ants to the filter paper balls was video-taped for 120 sec and analysed afterwards by counting the events of the different behaviours. Behaviour was considered as aggressive when ants touched the filter paper ball with both antennae and open mandibles or when they were biting into it. Antennation, that is, touching the filter paper ball with both antennae and closed mandibles, was considered as a nonaggressive behaviour.

The following test solutions containing mixtures of all four identified monoterpenes in hexane were prepared:

(1) mixture of α-pinene (3 mg), β-pinene (41 mg), myrcene (52 mg), and limonene (4 mg) in 100 mL hexane resembling the secretion of *Z. collaris*;

(2) mixture of α-pinene (24 mg), β-pinene (57 mg), myrcene (14 mg), and limonene (6 mg) in 100 mL hexane resembling the secretion of *Z. haworthi*.

Both mixtures contain terpenes in a total concentration of 1 μg/μL. For tests with 10 μg/μL, the mixtures were concentrated tenfold in a water bath. The relative concentrations of the single compounds matched the composition of the headspace analyses of the tergal gland secretion by GC/MS (Table 1). The concentration of either 1 μg/μL or 10 μg/μL is based on the assumption that the tergal gland reservoir of the two *Zyras* species is about 0.2 μL, equivalent to the volume of the similar sized *Aleochara curtula* Goeze [16] and that between 1/20 to 1/5 of the whole volume is released at one time.

2.4. Statistics. The results of the behavioural assays were analysed with the Mann-Whitney *U*-test using the software package STATISTICA 1999 Edition (StatSoft Inc., 1999).

3. Results

3.1. Chemical Analysis of the Tergal Gland Secretion. GC-MS analyses of volatiles released by *Z. collaris* and *Z. haworthi* revealed the presence of the monoterpenes α-pinene, β-pinene, myrcene, and limonene, which were identified by comparison of those of authentic reference samples (Figure 1, Table 1).

To compare the relative importance of each compound between the species, the relative proportions of the substances were calculated in accordance with [12]. This method reveals that *Z. haworthi* has a five times higher amount of α-pinene

than *Z. collaris* whereas the amount of myrcene in *Z. collaris* is approximately five times higher than in *Z. haworthi*. The amount of β-pinene and limonene is similar between the species.

3.2. Experiments on the Effect of the Tergal Gland Secretion. Filter paper balls treated with solutions mixed according to the results of the chemical analyses, representing the composition of the tergal secretion of *Z. collaris* and *Z. haworthi*, stimulated significantly more antennation by the ants than the control hexane. Furthermore, no significant aggression inducing effect was found (Figure 2).

4. Discussion

Using headspace SPME and GC-MS, the volatile compounds that were released by the two rove beetle species *Z. collaris* and *Z. haworthi* from their defensive tergal gland upon molestation were analysed. The analysis revealed the exclusive presence of the terpenes α-pinene, β-pinene, myrcene, and limonene. This is remarkable, because terpenes are absent from the tergal gland secretion of all the other 26 species from nine different tribes of this subfamily Aleocharinae which have been studied so far, including all the other species of the same tribe Myrmedoniini [8, 10, 16, 17]. Generally, the tergal gland secretion of the Aleocharine contains quinones as toxins, which are dissolved in alkanes, alkenes, aldehydes, ketones, acids, esters, and acetates [9]. Obviously, the composition of the secretion in the genus *Zyras* is unique within the subfamily.

This supports recent findings on the molecular phylogeny of Lomechusini [5], which show that the genus *Zyras* is much more distant to the genus *Pella* and that *Pella* should not be considered a subgenus of the former. This settles a long dispute on the phylogenetic relationship of these genera.

Due to the rarity of *Z. collaris* and *Z. haworthi*, the present study is based on the analysis of one specimen of each species only. So, it is not guaranteed that the mixtures found in the tergal glands of both specimens are representative of the entire species. Also possible methodological or sampling deviations cannot be excluded. However, in our earlier studies, we found that the qualitative composition of the defensive tergal gland secretion of the Aleocharinae is highly species specific and varies only quantitatively between individuals [9]. Thus, we consider that our results on the chemical composition of the tergal gland secretion are very likely to be valid. The uniqueness of the *Zyras* secretion within the Myrmedoniini is also supported by the fact that both *Zyras* specimens had qualitatively very similar secretions. Nevertheless, more studies on the chemical composition of the tergal gland secretion of *Zyras* species are required to substantiate our findings and to clarify the exact stereochemistry of the identified pinenes.

To study the role of the terpenes in the tergal gland secretion, the reaction of *L. fuliginosus* ants to mixtures of these compounds was studied in laboratory experiments. *L. fuliginosus* was chosen based on the literature where this species is described as host ant of *Z. haworthi* [13, 18] and because our *Z. haworthi* was collected in the vicinity of a nest of *L. fuliginosus*. This indicates that *L. fuliginosus* might be the host ant of *Z. haworthi,* whereas the host ants of *Z. collaris* remains unclear. Two different mixtures were tested, composed according to the ratio of single compounds in our chemical analysis of the secretion of both species. Mixtures were tested in two different concentrations covering the quantity of secretion released by the beetles under natural conditions. The experiments revealed no deterrent or aggression eliciting effect of these substances to the ants. Instead, increased antennation behaviour of ants towards filter balls treated with a mixture of these terpenes was observed. This reaction of the ants points to the fact that the terpenes might be used by the beetles to deal with their host ants in analogy to the ability of some myrmecophilous *Pella*-beetles, which repel aggressive host ants by the release of the ants' panic alarm pheromone sulcatone [8, 10]. However, none of the four identified monoterpenes have been described as pheromones in *L. fuliginosus* so far. Possibly, the antennation response of ants to the terpenes is based on their homobiosis with aphids. The aphids are protected by the ants, which receive the nutritious honeydew in return [6]. To obtain honeydew, ants antennate the aphid's abdominal tip. This behaviour strongly resembles the behaviour observed by us in interactions between myrmecophilous rove beetles and ants. In accordance with this idea, α-pinene, β-pinene, myrcene, and limonene have been reported to be present in some aphid species [19]. α- and β-Pinene as well as limonene occur in the aphid honeydew [20, 21]. Therefore, we hypothesise that these terpenes are used by ants to recognize aphids and that *Zyras* beetles mimic these compounds to calm down the aggressions of host ants during encounters. To address this hypothesis, it would be required (1) to unequivocally identify the host ants of both *Zyras* species, (2) to study in more details behavioural interactions between *Zyras* specimens and these host ants, (3) to identify aphid species that are relevant for the host ants, and (4) to examine the role of the identified terpenes on the interaction between these aphids and their host ants. This working plan is especially challenging because of the rarity of the beetles.

Taken together, the tergal gland secretion of *Z. collaris* and *Z. haworthi* is unique within the rove beetle subfamily Aleocharinae by its composition of the terpenes α-pinene, β-pinene, myrcene, and limonene. In biotests, *L. fuliginosus* ants were neither repelled nor did show aggressive behaviour towards these substances but were stimulated to antennation. Because terpenes are present in aphids, we hypothesize that *Zyras* beetles release these compounds to mimic aphids and achieve acceptance by their host ants.

Conflict of Interests

The authors declare that there is no conflict of interests.

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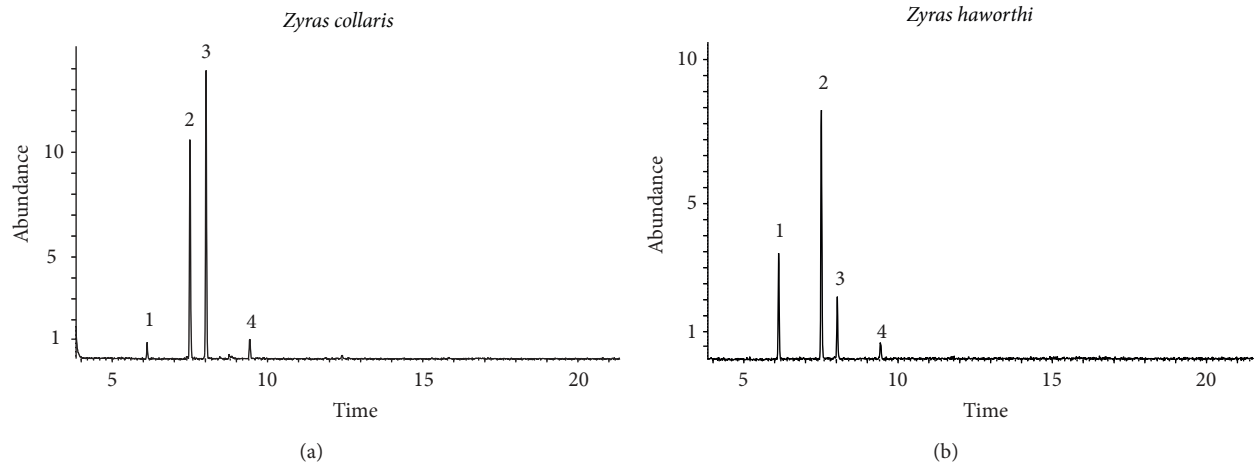

Figure 1: Gas chromatograms (TIC) of the tergal secretions obtained by stir bar irritation of *Zyras collaris* (a) and *Z. haworthi* (b). 1: α-pinene; 2: β-pinene; 3: myrcene; 4: limonene.

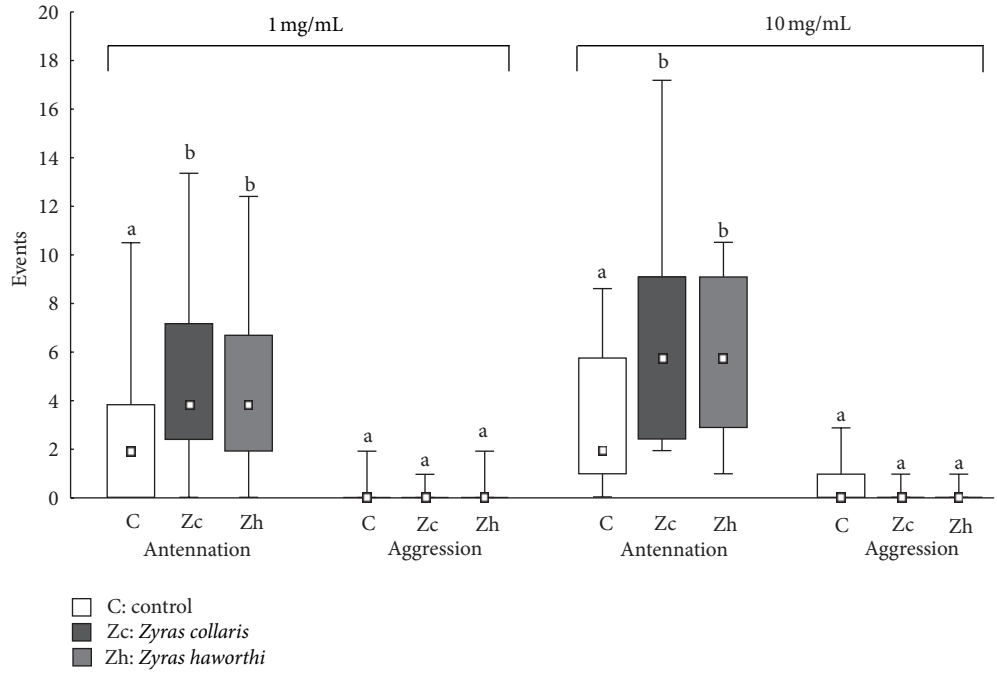

Figure 2: Antennation and aggressive behaviour (□: median, boxes: 25–75 percentiles, whiskers: min.–max.) by *Lasius fuliginosus* ants in a laboratory experiment towards a filter paper ball treated with mixtures of substances (1 mg/mL and 10 mg/mL), which are present in the tergal gland secretion of *Zyras collaris* and *Z. haworthi* rove beetles. Bars with different lower case letters are significantly different at $P \leq 0.05$ (Mann-Whitney U-test; control: $N = 40$; mixtures: $N = 20$).

reviewer carefully studied our paper and helped to improve it with his remarks.

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