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Multifunctional weaponry: The chemical defenses of earwigs

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ABSTRACT

Earwigs protect themselves against predators using pincer-like cerci and/or malodorous exudates secreted from abdominal glands. Little is known about the chemistry of these secretions and their potential functions. However, because earwigs live in aggregations and overwinter in soil, they are exposed to high microbial loads throughout their lifecycle, and we therefore hypothesized that the secretions are used not only to deter predators but also to combat pathogens and parasites in their environment. We analyzed the defensive secretions of the European earwig *Forficula auricularia*, the short-winged earwig *Apterygida media* and the woodland earwig *Chelidurella guentheri* by gas chromatography–mass spectrometry. The secretions of all three species contained 2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone, whereas *A. media* also produced 2,3-dimethyl-1,4-benzoquinone and 2-ethyl-3-methyl-1,4-benzoquinone. The latter has not been identified in the exudates of insects before. The composition and/or quantity of these components were species-specific and partially sex-specific. All secretions showed antimicrobial activity against Gram-positive and Gram-negative bacteria as well as two entomopathogenic fungi. Furthermore, the secretion of *F. auricularia* displayed nematicidal activity against *Caenorhabditis elegans*. Our data support the hypothesis that earwig secretions are multifunctional, serving both to deter predators and sanitize the microenvironment.

1. Introduction

Many insect orders have evolved exocrine glands to produce substances that protect them against predators (e.g., Cane, 1986; Huth and Dettner, 1990; Pavis et al., 1994; Osborne and Jaffe, 1998). The Dermaptera (earwigs) have evolved two strategies to avoid predation: when disturbed, they initially bend their abdomen forwards to use their pincer-like cerci as a mechanical defense, but if the disturbance persists they can also emit a malodorous defensive fluid from glands located in the third and/or fourth abdominal segments (Vosseler, 1890; Günther and Herter, 1974; Eisner et al., 2000). Such abdominal glands are present in numerous genera representing the families Labiidae, Chelisochidae and Forficulidae (Günther and Herter, 1974) and the glandular exudate is considered to be effective against a broad range of predators. Nevertheless, little is known about earwig chemical defenses. Only two species of earwigs, namely *Forficula auricularia* and *Doru taeniatum*, have been investigated in detail to determine the morphology of their defensive glands, the chemistry of the secretions and their biological functions (Vosseler, 1890; Eisner, 1960; Schildknecht and Weis, 1960; Eisner et al., 2000).

Earwigs live in dark and moist crevices, mostly in aggregations (Walker et al., 1993; Hehar et al., 2008). They are positively thigmotactic and therefore seek direct contact with surfaces and conspecifics (Weyrauch, 1929). Female earwigs also hibernate with their eggs in subterranean nests for several months (Günther and Herter, 1974). Groupliving and inter-individual contacts accelerate the transmission of pathogens and therefore of pathogen-mediated diseases (Schmid-Hempel and Schmid-Hempel, 1993; Côte and Poulin, 1995; Wertheim et al., 2005). With soil being the main reservoir for entomopathogenic fungi, bacteria and nematodes (Kung et al., 1990; Picard et al., 1992; Klingen and Haukeland, 2006), the habitat preferences and subsocial behavior of earwigs suggest they need effective protection not only from predators, but also a general defense against pathogenic microorganisms and parasites.

Chemical defenses are common among insects and complement their innate immune system (Vilcinskas, 2013). Some species are surrounded by clouds of their allelochemical exudates because gland depletion is not precisely regulated, or the secretion remains on the cuticle for some time after discharge caused by an attack (Dettner et al., 1992). Leaf beetle larvae are known to release antimicrobial substances from exocrine glands into their environment (Gross et al., 2008; Kirsch et al., 2011). The burying beetle *Nicrophorus vespilloides* produces oral and anal secretions consisting of

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a cocktail of volatiles that presumably contribute to both sanitation of the microenvironment and chemical preservation of the cadavers used as reproduction and breeding sites (Degenkolb et al., 2011). However, although the typical malodorous scent of the defensive secretion can be perceived wherever several earwigs aggregate (Vossler, 1890), neither the presence of the secretion in the headspace surrounding earwig aggregations nor its effect on potential pathogens or parasites have been evaluated.

Here we describe the first comparative survey of the chemical defense of earwigs, including the first compositional analysis of the defensive secretion of *Apterygida media* and *Chelidurella guentheri*. In addition, we tested the biological activity of the secretions on microorganisms using inhibition zone assays with Gram-positive and Gram-negative bacteria and two entomopathogenic fungi. The secretion of *F. auricularia* was also tested against the nematode *Caenorhabditis elegans*. The headspace surrounding individual specimens and natural aggregations of *F. auricularia* was analyzed using a needle trap device (NTD) to determine whether the bioactive substances are released into the microhabitat.

2. Materials and methods

2.1. Insects

Adult specimens of *F. auricularia*, *A. media* and *C. guentheri* were collected between June and September 2012 in Giessen, Germany. The earwigs were kept in plastic boxes in mixed-sex groups of 10–20 individuals, provided with apple pieces and water *ad libitum* and maintained for at least 5 days allowing defensive secretions to be replenished (Eisner, 1960). The insects were killed by freezing at −80 °C and were dissected immediately under a stereomicroscope to excise the glandular reservoirs prior to chemical and biological assays.

2.2. GC–MS Analysis

Excised glandular sacs from individual insects were transferred to 1.8-ml glass vials (CS – Chromatographie Service GmbH, Langerwehe, Germany) containing 100 µl (200 µl for *F. auricularia*) *n*-hexane (Sigma–Aldrich, St. Louis, USA). The sacs were ruptured with a sterile insect needle and sonicated in ice-cold water for 10 min. For quantitation, the solvent was spiked with 10 ng/µl *n*-octadecane (C_{18}, Sigma–Aldrich) as an internal standard. Five individual insects were analyzed per species and sex. One microliter of each extract was injected splitless into a gas chromatograph-mass spectrometer (GC–MS; CT 1128, Constellation Technology Corporation, Largo, Florida, USA) equipped with a VF-5MS column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness; Agilent Technologies, Inc., Santa Clara, USA). The temperature was programmed to rise from 50 °C (isothermic for the first 2 min) to 300 °C at 20 °C/min. The injection port was set to 230 °C and helium was used as carrier gas. Mass spectra were obtained in full scan electron ionization mode at 70 eV. Individual substances were identified by comparing their mass spectra (Supplementary Fig. S1) and retention indices with those of reference compounds (2-methyl-1,4-benzoquinone (MBQ); Sigma–Aldrich) or literature data (2,3-dimethyl-1,4-benzoquinone (DMBQ), 2-ethyl-3–methyl-1,4-benzoquinone (EMBQ); Machado et al., 2005; Föttinger et al., 2010). The defensive secretion of *Tribolium castaneum* was injected as an authentic reference for 2-ethyl-1,4-benzoquinone (EBQ) (Markarian et al., 1978). For data acquisition and integration of peak areas, the software MSD ChemStation E.02.00.493 (Agilent Technologies) was used. The statistical significance of observed quantitative differences was determined using SigmaStat 11.0 (Systat Software Inc., San Rose, USA). Data were compared pairwise using an unpaired *t*-test for normal distributions with equal variance, or otherwise using a *Mann Whitney U* test.

2.3. Antibacterial Activity

The activity of the exudates against standard Gram-positive and Gram-negative bacteria was assessed using *Micrococcus luteus* strain DSM 20030 (Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and the lipopolysaccharide-defective, streptomycin/ampicilin-resistant mutant of *Escherichia coli* K-12 strain D31 (Boman et al., 1974). The bacteria were cultured in LB (*E. coli*) or TSB broth (*M. luteus*) (Sigma–Aldrich). Fresh broth was inoculated with an aliquot of an overnight culture and grown to mid-log phase (OD_{600} = 0.5) at 37 °C (*E. coli*) or 30 °C (*M. luteus*). For the inhibition zone assays, 50 ml of broth containing 1% agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was inoculated with 200 µl (*E. coli*) or 500 µl (*M. luteus*) of the cultured bacteria and 7 ml of the inoculated agar medium was poured into Petri dishes (9 cm diameter).

2.4. Antifungal Activity

The antifungal activity of the secretions was evaluated using the entomopathogenic fungi *Beauveria bassiana* strain FSU 4404 (Jena Microbial Resource Collection, Jena, Germany) and *Metarhizium anisopliae* ARSEF 2038 (USDA – ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, USA). Both fungi are cosmopolitical and infect a wide range of insect species (Toledo et al., 2006), including earwigs (Günther and Herter, 1974). The fungi were cultured on 1.5% agar plates (Carl Roth) containing PD (*B. bassiana*) or YPG (*M. anisopliae*). When fully sporulating, conidia suspensions were prepared in 10 ml 0.9% NaCl, filtered through one layer of sterile Miracloth (Merck KGaA, Darmstadt, Germany) and adjusted with 0.9% NaCl to 1.3×10^7 spores/ml. For each assay, 70 ml agar was inoculated with 1 ml of the conidia suspension and poured into Petri dishes (9 cm diameter) in 7-ml aliquots.

2.5. General Bioassay Procedure

When solidified, seven 3-mm wells were punched into the agar plates and plugs were removed with a vacuum pump. The total quantity of benzoquinones produced by each species, as determined by chemical analysis (Table 1), was used as a guiding value for the concentrations applied in the inhibition zone assays. The defensive glands of seven individuals, separated by species and sex, were therefore pooled and diluted in *n*-hexane to obtain an average equivalent to one individual. Pure *n*-hexane was used as a negative control, and dilutions (0.1–12 µg/µl) of 1,4-benzoquinone (Fisher Scientifc GmbH, Schwerte, Germany) in *n*-hexane were applied as positive controls. We then added 3 µl per sample to each well and incubated the plates for 24 h at 37 °C (*E. coli*) or 30 °C (*M. luteus*). The Petri dishes inoculated with fungi were incubated for 72 h at 23 °C for *B. bassiana* and at 28 °C for *M. anisopliae*. After incubation the antimicrobial activity of the secretion was determined by measuring the inhibition zone diameter around each well. All tests were carried out in triplicate.

2.6. Nematicidal Activity

Due to the rare occurrence of *A. media* and *C. guentheri* specimens, nematicidal activity was tested using *F. auricularia* exudate only. The activity was tested against the model organism *C. elegans* strain N2 (kindly provided by Prof. Dr. U. Wenzel, Molecular Nutrition Research, Justus Liebig University Giessen, Germany) using two different approaches. The nematodes were cultured at room