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IS 7-HYDROXYPHTHALIDE A NATURAL COMPOUND OF OIL GLAND SECRETIONS? — EVIDENCE FROM *ARCHEGOZETES LONGISETOSUS* (ACARI, ORIBATIDA)

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ABSTRACT — The monophyly of a clade consisting of Astigmata and some of the glandulate Oribatida is supported by a synapomorphic set of five oil gland-derived secretion compounds (neral, geranial, neryl formate, 2-hydroxy-6-methylbenzaldehyde (=2,6-HMBD) and 2-formyl-3-hydroxybenzaldehyde (=γ-acaridial)), known as 'Astigmata compounds'. Another aromatic compound, 7-hydroxyphthalide, was reported for Astigmata and Oribatida, but is not known from any other source in nature. It was discussed whether this compound was a 'natural' part of oil gland secretions (and thus probably of phylogenetic significance) or an artifact. Here, we show that 7-hydroxyphthalide is the result of a post-extraction chemical transformation of γ-acaridial, and not a natural compound of oil gland secretions. We compared time series of raw extracts from *Archegozetes longisetosus* stored at -20°C with extracts stored at +23°C and show that storage at room temperature conditions promotes the transformation. However, since this reaction is quantitatively coherent, summing the amounts of both components seems to be a suitable approximation for the quantity of γ-acaridial in natural secretions, even if 7-hydroxyphthalide is found in the analyses.

KEYWORDS — γ-acaridial; 7-hydroxyphthalide; chemical ecology; artifact

INTRODUCTION

Most sarcoptiform mites possess a large pair of exocrine opisthonotal glands, commonly termed oil glands (Sakata and Norton, 2001). The chemical composition and biological function of secretions from these glands have been well studied in Astigmata since the 1970s (reviewed in Kuwahara, 2004), and substantial knowledge of oribatid mite oil gland chemistry has also accumulated in the last 15 years (e.g. Sakata *et al.*, 1995; Sakata and Norton, 2001; Raspotnig *et al.*, 2005, 2011; Takada *et al.*, 2005; Raspotnig, 2010). Oil gland secretion profiles are mostly species-specific and highly reproducible, so it has been suggested that these profiles might be a valuable set of characters for phylogenetic inference (Sakata and Norton, 2001).

It has been hypothesized that all oil-gland bearing sarcoptiform mites (Astigmata and Oribatida) form a monophyletic group (Norton, 1998), a hypothesis that is strongly supported by morphology and oil gland chemistry (e.g. OConnor, 1984; Sakata and Norton, 2001), but is controversially discussed on molecular basis (Domes *et al.*, 2007; Dabert *et al.*, 2010). The evolutionary origin (and

the sister group) of Astigmata lies, according to Norton's (1998) hypothesis, somewhere among the Desmonomata (more precisely: Malaconothroidea, =Trhypochthonioidea sensu Haumann, 1991). Investigations from the oil gland chemistry of these desmonomatan groups are thus of great interest.

More than one hundred chemical compounds are known from oil gland secretions of astigmatid and oribatid mites and include hydrocarbons, aromatics, terpenes and likely also alkaloids (Kuwahara, 2004; Raspotnig *et al.*, 2011). Compounds that are presumably synapomorphic for glandulate Oribatida and Astigmata have been termed 'Astigmata compounds' (Sakata and Norton, 2001), and include the monoterpenes neryl formate, neral and geranial, as well as the aromatics 2-hydroxy-6-methylbenzaldehyde (2,6-HMBD) and 2-formyl-3-hydroxybenzaldehyde (γ-acaridial). Numerous other compounds (e.g. saturated and unsaturated C13-C17 hydrocarbons) are also shared by Astigmata and Oribatida, but these have so commonly evolved independently among acarine and even arthropod exocrine gland secretions that they are of limited value for phylogenetic inference at the sarcoptiform level (they might represent plesiomorphic or homoplastic characters). However, a further aromatic compound, 7-hydroxyphthalide (7-hydroxy-3*H*-isobenzofuran-1-one), was found in extracts of the astigmatid mite *Oulenzia* sp. (Shimizu and Kuwahara, 2001) and also in the desmonomatan mite *Platynothrus peltifer* (Raspotnig *et al.*, 2005), but nowhere else in nature (Shimizu and Kuwahara, 2001). Raspotnig *et al.* (2005) concluded that 7-hydroxyphthalide could be an artifact in *P. peltifer* since it occurred in highly variable amounts, but Shimizu and Kuwahara (2001) explicitly termed it a natural compound of oil gland secretions. Recently, König (2010) observed 7-hydroxyphthalide in variable amounts also in oil gland secretions of *Trhypochthonius tectorum* (Trhypochthoniidae), from which it was unknown before (Sakata *et al.*, 2003; Raspotnig *et al.*, 2004; Heethoff *et al.*, 2011). Additionally, this component was detected in variable concentrations in *Perlohmannia* (Mixonomata) and some Histiostomatidae (Astigmata; Raspotnig, unpublished data).

These contradicting results were the motivation for the present study, which investigates whether 7-hydroxyphthalide is a natural compound of oil gland secretions (and thus might represent a sixth 'Astigmata compound' of phylogenetic significance) or simply an artifact. We therefore re-investigated the well-known oil gland secretion of *Archegozetes longisetosus* (Trhypochthoniidae; Sakata and Norton, 2003; Raspotnig and Föttinger, 2008; Heethoff *et al.*, 2011), for which 7-hydroxyphthalide has not been reported in the literature, but is observable in variable concentrations under some experimental conditions, as reported here.

MATERIALS AND METHODS

Specimens

Adult specimens from the laboratory strain *Archegozetes longisetosus* ran (Heethoff *et al.*, 2007) were used for whole body extractions. The culture was kept in constant dark at 28°C and saturated air humidity, with the unicellular alga *Chlorella* (as a commercially available powder) being provided as food and applied on filter paper every three days.

Extraction of oil gland secretion, storage and GC/MS analyses

Individual mites were carefully taken from the culture with a fine brush and transferred into 50 µL of hexane for three minutes to release oil gland secretions into the solvent. Afterwards, specimens were removed and 130 ng of 6-methyl-5-heptene-2-one (MHO, Sigma-Aldrich, Vienna) were added to the raw extract as an internal standard. Two different storage temperatures for the raw extracts were chosen: -20°C and +23°C. Raw extracts (2 µL, containing ≈5 ng MHO) were analyzed after 1, 2, 4, 15, 18 and 24 hours. Samples were taken from the -20°C freezer only for removing the 2 µL for analyses, and placed back immediately afterwards.

A trace gas chromatograph (GC) coupled to a DSQ II mass spectrometer (MS; both from Thermo, Vienna, Austria) and equipped with a ZB-5MS fused silica capillary column (30 m length, 0.25 mm

diameter, 0.25 μm film thickness, Phenomenex, Germany) was used for the analyses. Injection was splitless with helium (at a constant flow rate of 1.2 ml/min) as a carrier gas. The column temperature was programmed from 50°C (held for 1min) to 200°C at 10°C/min, and then to 300°C at 15°C/min. The ion source of the mass spectrometer and the transfer line were kept at 150°C and 310°C, respectively. Electron impact (EI) spectra were recorded at 70 eV.

Determination of relative compound abundance was based on the integration of peak areas in the chromatograms, and thus expressed in % peak area of whole secretion (WS). Absolute amounts of components were calculated relative to a constant concentration of an internal standard (5 ng MHO, see above), and thus expressed in % peak area of this standard.

RESULTS

Chromatograms taken immediately after extraction showed a constant profile of 11 compounds (Figure 1). These consisted of the 9 components already described by Sakata and Norton (2003), the addi-

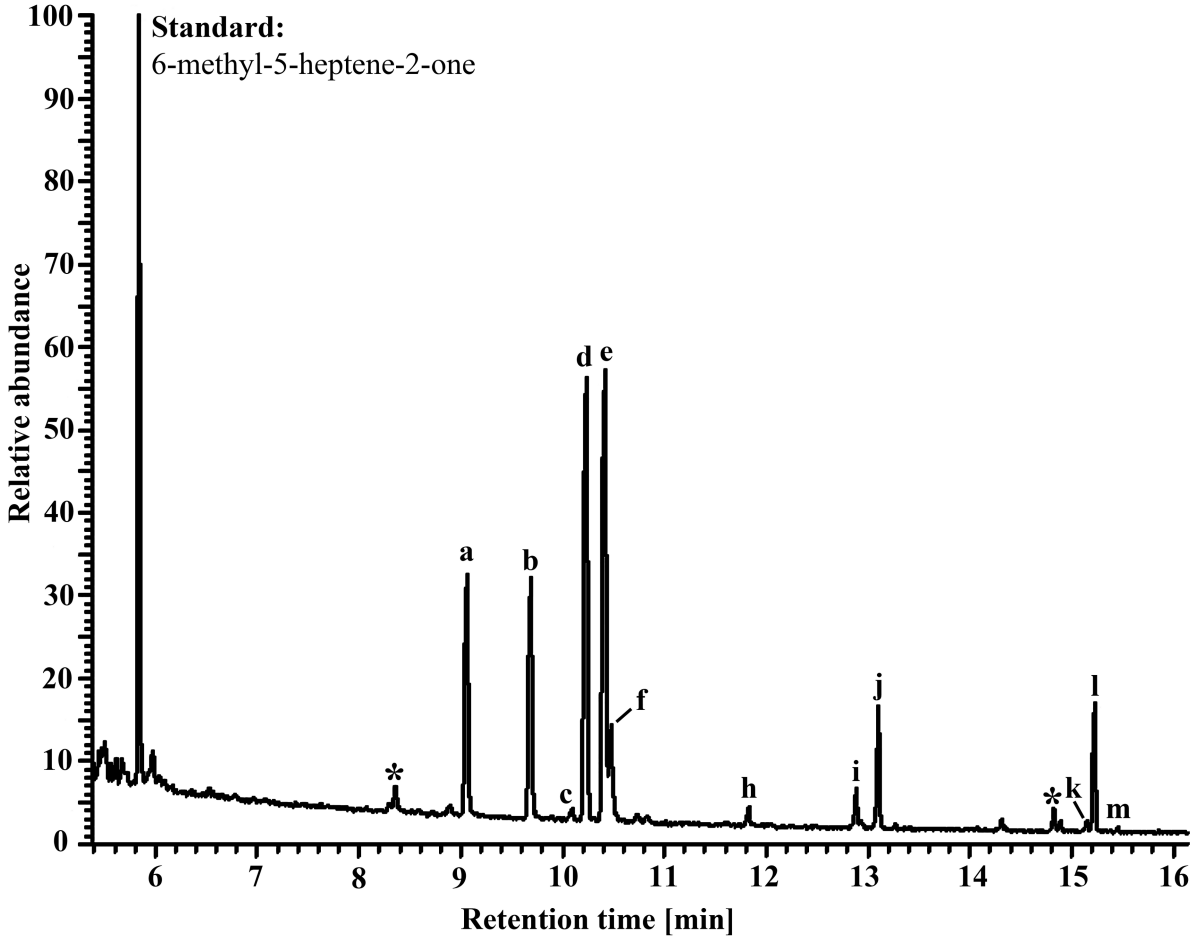

FIGURE 1: Gas chromatographic pattern of components, 24 hours after extraction, stored at -20°C. This pattern is identical to chromatograms taken directly after extraction. The amount of the internal standard (6-methyl-5-heptene-2-one) corresponds to 5ng. Peaks: a: 2,6-HMBD; b: neral; c: geranial; d: neryl formate; e: γ-acaridial; f: tridecane; h: tetradecane (this in an artifact of unknown origin); i: pentadecene; j: pentadecane; k: heptadecadiene; l: heptadecene; m: heptadecane. The * peaks arise from contaminations of the capillary column.

tional heptadecane found by Raspotnig and Föttinger (2008) and tridecane as a newly discovered component.

In detail, compounds were identified as: 2,6-HMBD (=2-hydroxy-6-methyl-benzaldehyde, peak a, 13% of whole secretion (=WS)), neral (peak b, 13% of WS), geranial (peak c, 1% of WS), neryl formate (peak d, 25% of WS), γ-acaridial (=3-hydroxybenzene-1,2-dicarbaldehyde; peak e, main component with 29% of WS), tridecane (peak f, 5% of WS), pentadecene (peak i, 3% of WS), pentadecane (peak j, 6% of WS), heptadecadiene (peak k, 1% of WS), heptadecene (peak l, 5% of WS) and heptadecane (peak m, <1% of WS). The absolute amount of secretion averaged 368% ($\pm$2%, n=6) of the standard, corresponding to 18.4ng of MHO (which does not imply that the quantitative amount of secretions is also 18.4ng). Relative (with respect to other compounds in the secretion) and absolute (with respect to the standard) amounts of secretions and compounds did not vary during the 24h storage at -20°C. All six replicates (1h, 2h, 4h, 15h, 18h, 24h) had identical chromatograms (Figure 1 shows a chromatogram taken after 24h at -20°C). None of the chromatograms had a peak corresponding to 7-hydroxyphthalide.

When stored at +23°C, however, a peak with a retention time of 11.60 min., and a mass spectrum corresponding to 7-hydroxyphthalide (Figure 2, peak g and Figure 3), appeared already after 1

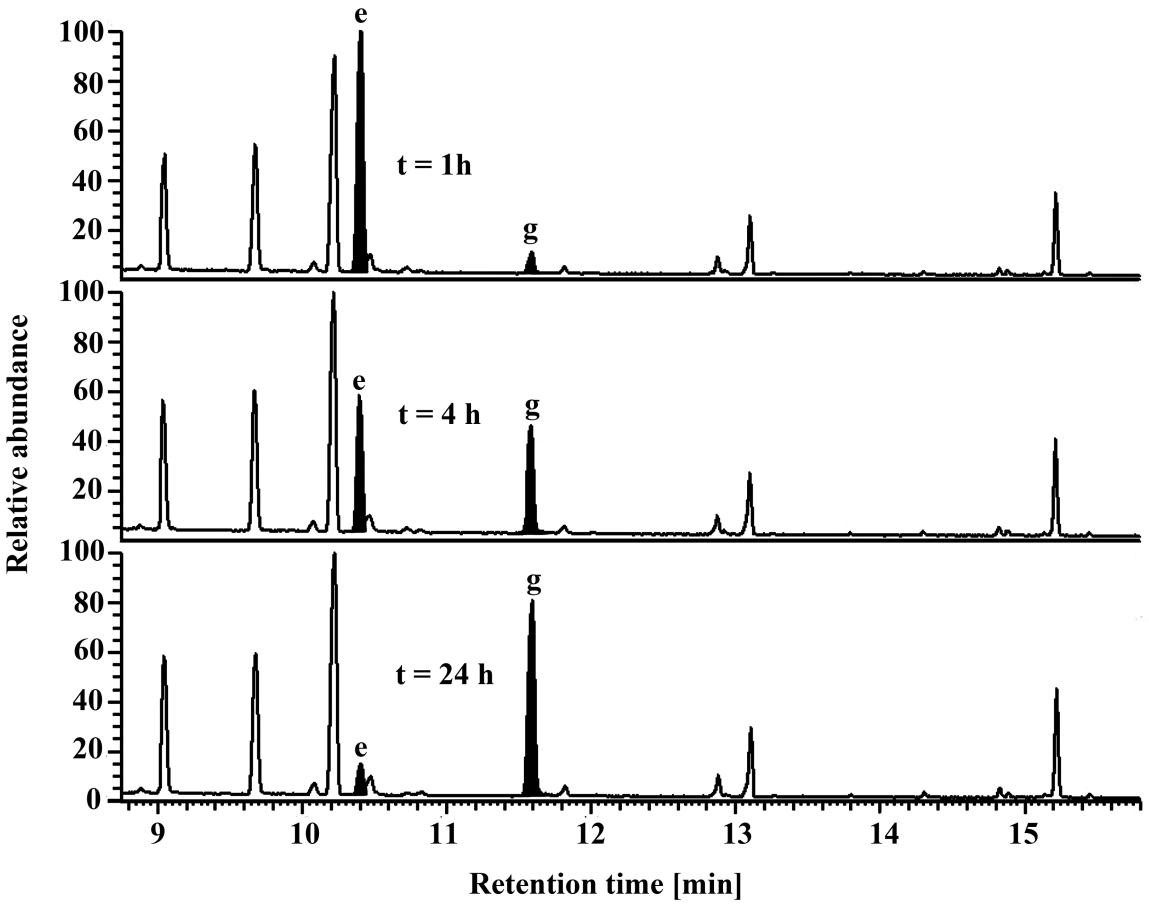

FIGURE 2: Gas chromatographic patterns measured from a raw extract stored at 23°C, 1 hour, 4 hours and 24 hours after extraction. Peak e (γ-acaridial) decreases during time course while peak g (7-hydroxyphthalide) grows. All other peaks remain quantitatively stable.

hour and became more and more prominent over time (starting with 3% of WS after 1 hour and ending up with 25% of WS after 24 hours; Figures 2 and 4).

At the same time, the abundance of γ-acaridial decreased (starting with 25% of WS after 1 hour and ending up with 3% after 24h). After 24 hours, 7-hydroxyphthalide replaced most of the γ-acaridial. The total amount of both components together, however, resembled the starting amount of γ-acaridial (Figure 4) - the amounts of both components were strongly correlated (y=-0.99+28.6, R^2=0.999), indicating that the occurrence of peak g was a direct result of the disappearance of peak e.

Mass spectra of γ-acaridial and 7-hydroxyphthalide differ only slightly (Figure 3) and were found identical to those shown by Shimizu and Kuwahara (2001). Both spectra have their M^+ at *m/z* 150 and the base ion at *m/z* 121, followed by diagnostic ions at *m/z* 122 (in acaridial: M^+-CO;

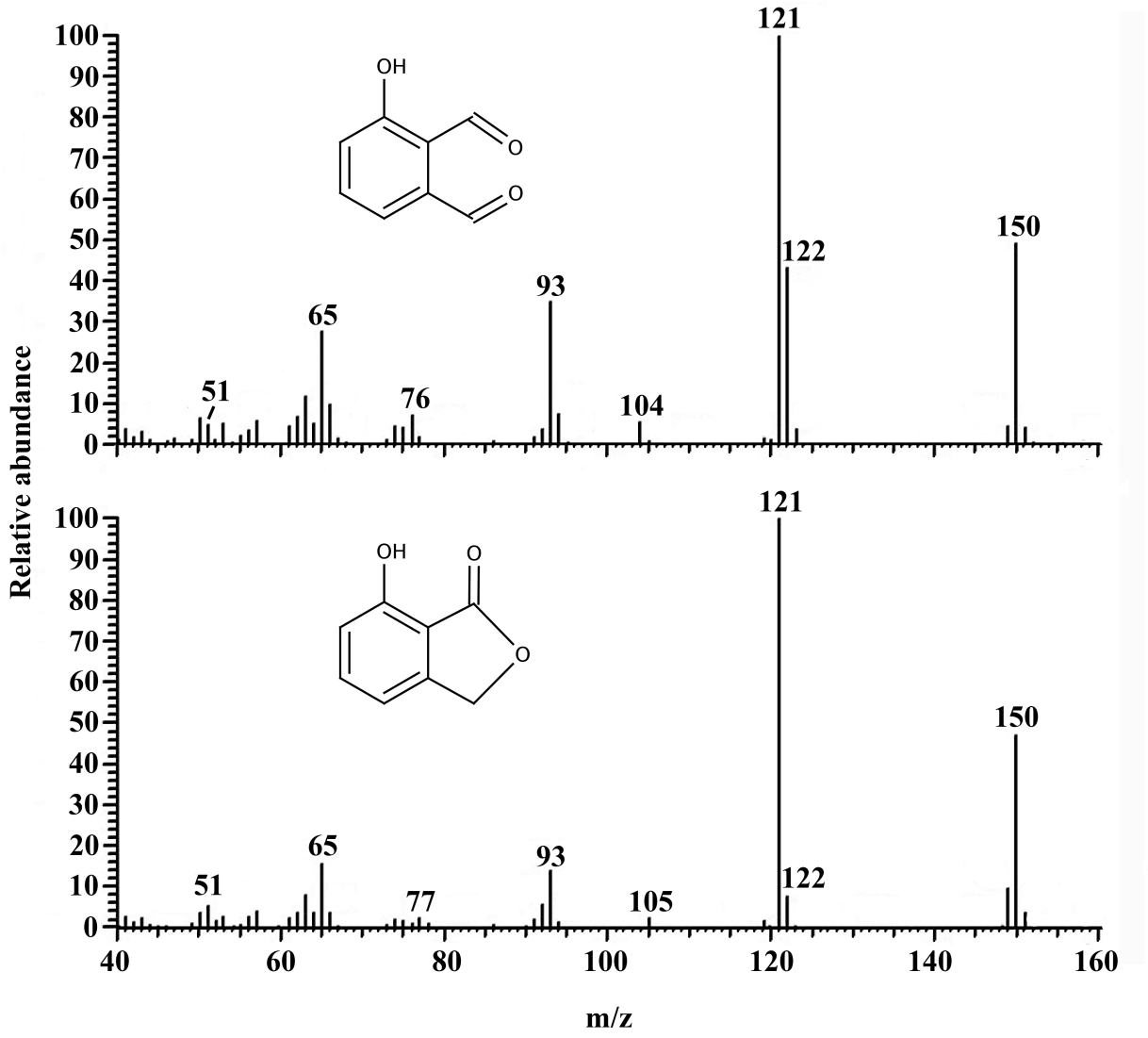

FIGURE 3: Mass spectra of γ-acaridial (above) and 7-hydroxyphthalide (below), both with M^+ at *m/z* 150. Note differences at peaks *m/z* 76/77, *m/z* 104/105, and relative differences at *m/z* 122.

in the phthalide: isotopic peak with relative intensity of 7.7% of the base ion $C_7H_5O_2$, indicating the presence of 7 carbon atoms), *m/z* 104(acaridial) / 105(phthalide), *m/z* 93, *m/z* 76(acaridial) / 77(phthalide), *m/z* 65 and *m/z* 51. A detailed description of mass spectra and fragmentation of both components is given in Shimizu and Kuwahara (2001) and Sakata and Kuwahara (2001) and will not be repeated here.

DISCUSSION

The chemical profile of oil gland secretions from *A. longisetosus* shown here adds a new compound (tridecane) to the profiles published earlier (Sakata and Norton, 2003; Raspotnig and Föttinger, 2008). This compound may have been masked in earlier investigations by γ-acaridial since retention times were quite close to each other under the chromatographic conditions used, as was shown for *Collohmannia gigantea* (Raspotnig and Föttinger, 2008).

Is 7-hydroxyphthalide a natural compound of oribatid and astigmatid oil gland secretions and thus perhaps another 'Astigmata compound'? Probably not. While Shimizu and Kuwahara (2001) found it in low concentrations co-occurring with γ-acaridial and termed it a 'natural' compound of the astigmatid *Oulenzia* sp., Raspotnig et al. (2005) were probably right in hypothesizing that it is an artifact, however, they did not suggest any mechanistic explanations for the sporadic occurrence of this compound. Where observed, 7-hydroxyphthalide always co-occurs with γ-acaridial: i.e. in Astigmata (*Oulenzia*: Shimizu and Kuwahara, 2001; Histiostomatidae: Koller, Wirth, Raspotnig, unpublished); Oribatida: several *Trhypochthonius* species (König, 2010; Raspotnig, unpublished); *Perlohmannia* (Raspotnig, unpublished); *Platynothrus* (Raspotnig *et al.*, 2005) and *Archegozetes* (this study).

We showed here that the occurrence of 7-hydroxyphthalide can be induced within an hour by storing the γ-acaridial-containing raw extracts outside the freezer at +23°C, and that the relative amount of 7-hydroxyphthalide depends only on the time in which the raw extracts are not stored at low temperatures. This well explains the variable amounts of 7-hydroxyphthalide found by Raspotnig et al. (2005) and the other above-mentioned authors since the duration of room-temperature storage prior to analysis is usually not documented.

When combined, γ-acaridial and 7-hydroxyphthalide showed constant relative adundance in the oil gland secretions, and no other compound was quantitatively affected by storage at 23°C (Figure 2). Hence we suggest for quantitative investigations on γ-acaridial-containing secretions

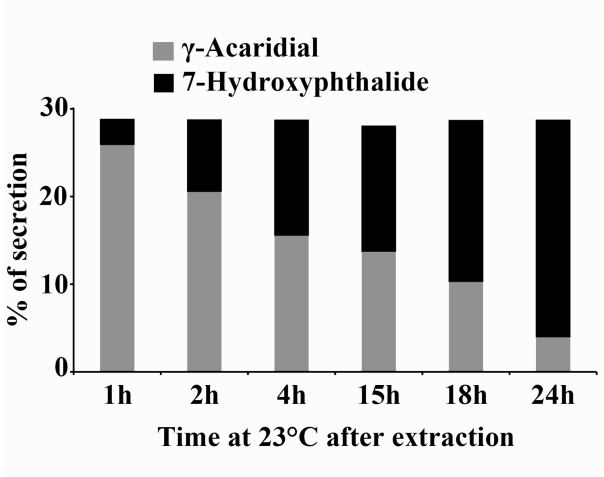

FIGURE 4: Comparison of amounts of γ-acaridial and 7-hydroxyphthalide during the 24 hour time course at 23°C. When both are combined, the relative amount in the whole secretion remains stable (also documented by a linear regression with y=-0.99+28, R^2=0.999).

to always add the peak area of 7-hydroxyphthalide (if it occurs) to the area of γ-acaridial - this should correspond to the amount of γ-acaridial originally present in the fresh raw extracts (Figure 4).

We cannot exclude the possibility that 7-hydroxyphthalide is also a 'natural' compound of oil gland secretions in other species, but we think this is unlikely. For all oribatid mite species where it was observed so far, most of the profiles were found not to contain 7-hydroxyphthalide, and otherwise had very stable secretion profiles (Raspotnig 2010). Additionally, differing from Raspotnig et al. (2005), Raspotnig and Föttinger (2008) found no indication of 7-hydroxaphthalide in repeated measurements of extracts from *Platynothrus peltifer* (which were done immediately after extraction). Also in Histiostomatidae, 7-hydroxyphthalide only occurs in some extracts and does not belong to the 'natural' compounds (Koller, Raspotnig and Wirth, unpublished).

Most convincingly it is easily possible to induce the presence of 7-hydroxyphthalide in originally 7-hydroxyphalide-free but γ-acaridial-containing extracts simply by changing the storing conditions to higher temperatures. Shimizu and Kuwahara found both, γ-acaridial and 7-hydroxyphthalide only in trace quantities (see Figure 1 of Shimizu and Kuwahara, 2001). The extraction and analytical procedure of Shimizu and Kuwahara (2001; 3 min in hexane) was similar to that used in our study, so the occurrence of 7-hydroxyphthalide in *Oulenzia* sp. was probably also an artifact.

Shimizu and Kuwahara (2001) already proposed that an intramolecular Cannizzaro reaction with hydride transfer is responsible for the transformation of γ-acaridial to 7-hydroxyphthalide, but since they termed it a 'natural' compound we assume they considered this reaction to be a part of the biochemical synthesis-pathway. We propose that this transformation occurs after extraction, following a reaction scheme similar to the intramolecular conversion of 1,4-dialdehydes to γ-lactones (Bergens *et al.*, 1990). While the Cannizzaro-reaction is usually base-promoted (Cannizzaro 1853), and the intramolecular reaction benefits from some catalysis in methanol solution (Bergens *et al.*, 1990), we found it to occur quite fast at room temperature (and even much faster at 30-35°C, data not shown) in the hexane raw extracts. Unraveling the detailed chemical reaction of this transformation is beyond the scope of this study, but might also be of economical interest: 7-hydroxyphthalide is known to be effective against antigenic diseases like rheumatism (Shimizu and Kuwahara, 2001). Hence, this may add another interesting aspect to the well-known anti-fungal, bacteriostatic, defensive and pheromonal properties of sarcoptiform oil gland secretions (Raspotnig, 2010; Raspotnig *et al.*, 2011).

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