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Naphthoquinones and Anthraquinones from Scent Glands of a Dyspnoid Harvestman, *Paranemastoma quadripunctatum*

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Abstract Extracts of *Paranemastoma quadripunctatum* (Opiliones, Dyspnoi, Nemastomatidae) contained seven components, all of which likely originated from the secretion of well-developed prosomal scent glands. The two main components (together accounting for more than 90% of the secretion) were identified as 1,4-naphthoquinone and 6-methyl-1,4-naphthoquinone. The minor components were 1,4-naphthalenediol, two methoxy-naphthoquinones (2-methoxy-1,4-naphthoquinone, and 2-methoxy-6-methyl-1,4-naphthoquinone) and two anthraquinones (2-methyl-9,10-anthraquinone and a dimethyl-9,10-anthraquinone). While some chemical data on scent gland secretions of the other suborders of Opiliones (Cyphophthalmi, palpatorean Eupnoi, and Laniatores) already exist, this is the first report on the scent gland chemistry in the Dyspnoi. Naphthoquinones are known scent gland exudates of Cyphophthalmi and certain Eupnoi, methoxy-naphthoquinones and anthraquinones are new for opilionid scent gland secretions.

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Introduction

Large prosomal sac-like glands—so-called repugnatorial or scent glands—represent the main exocrine system in harvestmen, and they are a synapomorphic character of all Opiliones. These glands typically open to the body outside via one large orifice on the lateral margins of the carapace, either dorsal to coxae I (Palpatores), dorsal to coxae II (Laniatores), or atop tubercles ("ozophores") that protrude from the prosoma (Cyphophthalmi). Scent glands are primarily thought to serve chemical defense (e.g., Martens 1978) although, in certain groups, additional functions may have evolved (Holmberg 1986; Machado et al. 2002). Since the 1950s, the chemistry of scent gland secretions of various opilionids has been investigated sporadically, nevertheless leading to a considerable amount of chemical data (review in Gnaspini and Hara 2007): Specific sets of benzoquinones and phenols occur in gonyleptoid Laniatores (Grassatores) (e.g., Hara et al. 2005), while terpenes, esters, and nitrogen-containing compounds have been found in one representative of travunioid Laniatores (Insidiatores) (Ekpa et al. 1984). In the Palpatores, chemical knowledge on scent glands is biased: In Eupnoi (at least in some Sclerosomatidae), acyclic components such as ethyl ketones and their corresponding alcohols are common (Ekpa et al. 1985), while naphthoquinones were reported from Phalangiidae (Wiemer et al. 1978). Recently, both acyclic ketones and naphthoquinones were also detected in Cyphophthalmi (Raspotnig et al. 2005). By contrast, data on the chemical composition of scent gland exudates in the Dyspnoi are lacking.

Dyspnoid scent glands generally are more inconspicuous and cryptic than in other groups and show many aberrant features. Ozopores may be hidden or covered (instead of being exposed, as characteristic for defensive glands), and in the majority of Dyspnoi, noticeable emission of secretion from ozopores, as occurs in other Opiliones, does not occur at all. In some taxa, scent glands obviously produce and store solid secretion; this may be true for Ischyropsalididae (Juberthie et al. 1991) and Trogulidae (Schaider and Raspotnig 2009). In these cases, the emission of secretion is thought to be gaseous, by slow sublimation of solid glandular contents (Juberthie et al. 1991). In species of *Trogulus*, however, emission has never been detected, not even under heavy mechanical disturbance (Pabst 1953). Scent gland features in Dyspnoi have led to doubts concerning their defensive nature (Schaider and Raspotnig 2009), with the chemistry of secretion remaining a major open question. Since scent gland secretions and their chemical profiles, respectively, also seem to constitute phylogenetically useful information (e.g., Roach et al. 1980; Hara et al. 2005), and since the relationship of Palpatores and Laniatores is still uncertain (Shultz and Regier 2001; Giribet et al. 2002), the scent gland chemistry of Dyspnoi may help clarify phylogenetic questions.

We here present the first chemical analysis of a dyspnoid scent gland secretion using the model nemastomatid species, *Paranemastoma quadripunctatum*.

Methods and Materials

Specimens of *Paranemastoma quadripunctatum* (Perty 1833) (Opiliones, Dyspnoi, Nemastomatidae) were collected by hand in Styria and Carinthia (Austria). Extraction of scent gland secretions was performed from living specimens. In total, individual extracts from 18 adults of both sexes were prepared by two methods: 1) by dabbing scent gland secretion on filter paper pieces that were subsequently extracted in 100 µl of hexane for 10 min (8 extracts); 2) by whole body extraction of individuals in 150 µl of hexane or ethyl acetate for about 30 min (10 extracts). With respect to the latter method, in 8 of 10 cases, the solvent was heated to 60°C and sonicated to dissolve the components. Aliquots of extracts (2 µl) were subject to gas chromatographic-mass spectrometric (GC-MS) analysis, using a Trace GC2000 coupled to a Voyager MS (both from Thermo, Vienna, Austria). The GC was equipped with a ZB-5MS fused silica capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness, Phenomenex, Germany). Injection was splitless with helium (at 1.5 ml min^{-1}) as carrier gas. The column temperature was programmed from 50°C (held for 1 min) to 200°C at 10°C min^{-1}, and then to 300°C at 15°C min^{-1}. The ion source of the MS and the transfer line were kept at 170°C and 310°C, respectively. Electron impact (EI) spectra were recorded at 70 eV.

Reference compounds for comparisons of GC-MS data were purchased from Sigma (Vienna, Austria) (1, 4-naphthalenediol, 1,4-naphthoquinone, 2-methoxy-1,4-naphthoquinone, 2-methyl-9,10-anthraquinone). For authentic 6-methyl-1,4-naphthoquinone, we used a natural source, namely the scent gland secretion of another opilionid, *Phalangium opilio*, in which 6-methyl-1,4-naphthoquinone was identified by both MS and NMR-studies (Wiemer et al. 1978).

Results

Chemical Identification of Extract Components Seven components (A–G) were detected in the extracts by GC (Fig. 1; Table 1). The two major compounds (A and C), together accounting for about 90% of the extract profile, were known previously from a study with the cyphophthalmid harvestman *Cyphophthalmus duricorius* (Raspotnig et al. 2005), and appeared to be 1,4-naphthoquinone and 6-methyl-1,4-naphthoquinone, respectively. For final identification, we compared both their MS patterns as well as GC retention times to those of the authentic standards, finding full correspondence. The remaining minor components were identified either: 1) by comparison of mass spectra and retention times to commercially available compounds (compounds B, D, and F) or 2) tentatively only, by interpretation and comparison of mass spectra to spectra from the NIST-library (compounds E and G) (Table 1).

The mass spectrum of compound B clearly was related to that of compound A (1,4-naphthoquinone), and was identified as 1,4-naphthalenediol. Compounds D and E also appeared to be homologous naphthoquinones, namely methoxy-naphthoquinones. Compound D was identified as 2-methoxy-1,4-naphthoquinone (M^+ at *m/z* 188). Compound E exhibited a comparable mass spectrum, but with fragment ions in the higher mass range, all shifted 14 mass units higher (M^+ was at *m/z* 202), indicating the presence of an extra methyl-group. The position of the additional methyl-group in compound E was not determined but, by analogy to component C (6-methyl-naphthoquinone), we tentatively identify this compound as 2-methoxy-6-methyl-1,4-naphthoquinone. Components F and G are anthraquinones, namely a 2-methyl-9,10-anthraquinone (M^+ at *m/z* 222; component F), and a homologous component that again differs only by the presence of an additional methyl-group (M^+ at *m/z* 236; compound G). The latter compound is probably a dimethyl- or an ethyl-anthraquinone.

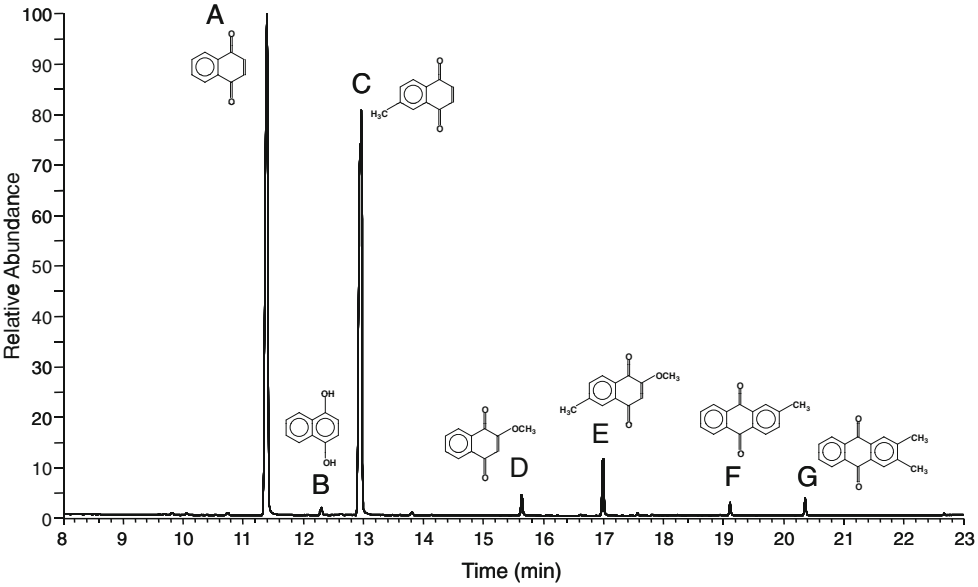

Fig. 1 Typical gas chromatographic profile of a droplet loaded with scent gland secretion dabbed onto filter paper from the mouth of a *Paranemastoma quadripunctatam* male. Components: Peak A (1,4-naphthoquinone), B (1,4-naphthalenediol), C (6-methyl-1,4-naphthoquinone), D (2-methoxy-1,4-naphthoquinone), E* (2-methoxy-6-methyl-1,4-naphthoquinone), F (2-methyl-9,10-anthraquinone), G* (2,3-dimethyl-9,10-anthraquinone). [Components E and G, marked with an asterisk were only tentatively identified (see text).]

Chromatographic Profiles and Secretory Glands The compounds found in the extracts are most likely from the scent glands that open to the body outside via one large ozopore on either side of the prosoma in the region of coxae I. In whole body-extracts obtained from short extraction times (30 min) at room temperature and hexane or ethyl acetate as a solvent, the chemical profile observed from filter-paper extraction was detected only in two out of 10 individuals. The remaining eight individuals (where no secretion was detected) were extracted again in hot hexane for 1 h, followed by ultrasonic treatment (10 min), and these extracts contained the same set of components as in filter-paper extracts, but in reduced amounts. In particular, the anthraquinones were found present only in trace amounts. In order to link unequivocally the extracted components with the scent glands, living specimens were squeezed gently with a forceps, which led to the emission of a droplet of clear enteric fluid from the mouth. Subsequently, and beginning from the lateral sides of the droplet, color was mixed into the enteric fluid, until the whole droplet became brownish. GC profiles of filter-paper extracts of this droplet clearly showed the full chemical profile of all seven components as described above. Such droplets were dabbed from eight individuals, and each exhibited a consistent chemical profile corresponding to the profiles obtained from whole body-extracted individuals (Fig. 1). No differences in secretion profiles were recognized between males and females. If clear droplets were examined (dabbed from

Table 1 Gas chromatographic—mass spectrometric identification of secretion components in *Paranemastoma quadripunctatum*

Peak	RT (min)	Rel. abund. (in % peak area)	Fragmentation pattern m/z (relative intensity)	Identified as
A	**11.39**	**49**	159 (12), 158 (100), 130 (70), 104 (75), 102 (78), 76 (68), 75 (23), 74 (21), 66 (11), 51 (16), 50 (35)	**1,4-naphthoquinone**
B	12.31	< 1	161 (9), 160 (88), 132 (15), 131 (19), 105 (27), 104 (100), 77 (13), 76 (41), 57 (18), 50 (21)	1,4-naphthalenediol
C	**12.97**	**43**	173 (15), 172 (100), 157 (13), 144 (45), 118 (61), 116 (58), 115 (63), 90 (28), 89 (43), 63 (19)	**6-methyl-1,4-naphthoquinone**
D	15.64	1	189 (12), 188 (100), 173 (37), 160 (36), 159 (41), 158 (45), 132 (11), 131 (19), 130 (30), 104 (20), 102 (81), 101 (17), 89 (89), 76 (42), 69 (23), 50 (26)	2-methoxy-1,4-naphthoquinone
E	17.00	4	203 (13) , 202 (100), 187 (34), 174 (26), 173 (48), 172 (33), 146 (6), 145 (14), 144 (17), 131 (15), 118 (13), 116 (54), 115 (40), 103 (76), 89 (29), 77 (27), 69 (13), 63 (17)	2-methoxy-6-methyl-1,4-naphthoquinone*
F	19.11	< 1	223 (16), 222 (100), 221 (14), 207 (17), 194 (44), 193 (13), 166 (33), 165 (92), 164 (17), 163 (14), 139 (11), 115 (6), 97 (9), 89 (7), 82 (29)	2-methyl-9,10-anthraquinone
G	20.36	1	237 (18), 236 (100), 235 (10), 221 (31), 208 (33), 207 (12), 193 (17), 180 (8), 179 (17), 178 (24), 165 (54), 152 (9), 139 (4), 104 (10), 89 (23), 76 (12)	dimethyl-9,10-anthraquinone*

Components in bold are main components of the secretion; components marked with * have been identified tentatively (on the basis of their mass spectra) only. Quantification of components is based on the relative abundance of peak areas (whole secretion=total of all peak areas = 100%).

the mouth before the colored secretion was mixed in), none of the profile-components was detected.

Discussion

As outlined in the introduction, problems in extracting dyspnoid secretions are due to: 1) their possibly solid nature and, 2) the general reluctance of Dyspnoi to release secretions. Thus, after several previous unsuccessful attempts to extract and analyze secretions of some Dyspnoi (e.g., species of *Trogulus* and *Anelasmocephalus*), we present here the first example of dyspnoid scent gland chemistry. Behavioral experiments with *P. quadripunctatum* could not easily evoke emission of secretion; in fact, observable discharge of secretion from ozopores (common in many other Opiliones) did not occur. In case of disturbance, however, individuals of *P. quadripunctatum* regurgitate a droplet from the mouth, which subsequently is mixed with scent gland secretion, indicated by a gradual color change of the droplet from clear to brownish. A similar situation is known for certain Laniatores where a regurgitated droplet runs from the mouth to the ozopores, mixing with scent gland secretion, thereby also gradually changing color (Eisner et al. 2004). In *P. quadripunctatum*, we might have observed the reversed emission sequence; secretion from scent glands may run towards the mouth region where it is dissolved in a regurgitated droplet of enteric fluid. Another possibility is that regurgitated enteric fluid runs first to the ozopores, partly dissolving (viscous or even solid) scent gland exudates. Subsequently, the scent gland secretion-enteric fluid mixture may run back to the mouth, finally mixing with more enteric fluid, thus creating an applicable defensive droplet. Since the chemistry indicates a viscous or partly solid exudate, the latter scenario may be the most probable. In either case, secretion or a secretion-fluid-mix reaches the regurgitated droplet at the mouth from both sides at the same time, as indicated by the change of color beginning at the lateral margins of the oral droplet. Additionally, the location of ozopores is consistent with this view; i.e., ozopores in *P. quadripunctatum* (dorsal to coxae I as in all Palpatores) are hidden beneath a dorso-lateral fold of the carapace, and are directed ventrally. Thus, secretion, or a secretion-enteric mixture, is discharged towards coxae I, and flows ventrally towards the mouth. The scent gland secretion-enteric fluid mixture in *P. quadripunctatum* may be applied to a potential predator with the chelicerae. As such, application of secretion may represent a special kind of "leg dabbing" as known from Cyphophthalmi and certain laniatoreans (Juberthie 1961; Eisner et al. 2004).

Furthermore, the anthraquinones found in the *P. quadripunctatum* exudate are highly unusual compounds for exocrine secretions of arthropods, since these are solid, poorly soluble compounds under normal ambient temperatures. Thus, dissolving or diluting scent gland exudates in enteric fluid may be necessary in order to release secretion quickly. The rapid solubility of the scent gland exudates of *P. quadripunctatum* in enteric fluid is, nevertheless, remarkable because attempts to extract these exudates from whole bodies by using different solvents at elevated temperature with sonication did not produce concentrated extracts. In the rare cases where we found large amounts of the scent gland components in whole-body extracts, we suppose that we extracted individuals at the very moment of "loading" droplets of enteric fluid with scent gland secretion. Large amounts of these components were detected in droplets of both sexes, excluding the participation of male cheliceral glands as a possible source of chemical material (these glands are present in male nemastomatids, see e.g., Martens and Schawaller 1977).

We have preliminary data that the use of enteric fluid to dissolve (viscous or even solid) scent gland exudates may be characteristic of Dyspnoi. Even in trogulids and ischyropsalidids, this mechanism of secretion release appears to be more likely than the hitherto proposed slow sublimation of solid scent gland contents (e.g., Juberthie et al. 1991). In any case, the *Paranemastoma* exudate may represent a "prestage" to these completely solid secretion boli in the groups mentioned above. From a chemosystematic point of view, anthraquinones and methoxy-naphthoquinones are new for opilionid scent gland secretions but are expected to occur in other Dyspnoi as well. By contrast, 1,4-naphthoquinone and 6-methyl-1,4-naphthoquinone (the two main components of the *Paranemastoma* exudate) may be widespread, and are previously known from scent glands of Cyphophthalmi (Raspotnig et al. 2005), and from glands of at least one representative of Eupnoi, *Phalangium opilio* (Wiemer et al. 1978).

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